

KERATIN SYNTHESIS DURING DEVELOPMENT OF THE EMBRYONIC CHICK FEATHER

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ABSTRACT

The synthesis of keratin proteins during development of the embryonic chick feather was studied by quantitative gel electrophoresis of the reduced and carboxymethylated proteins. The results demonstrated a coordinated synthesis of the major keratin proteins, during and after the onset of keratin synthesis. The results from gel electrophoresis correlated well with electron microscope visualization of keratin fibrils in the developing feathers. Autoradiography at the electron microscope level indicated that the feather cells lose the ability to synthesize DNA before keratin synthesis begins, but retain the ability to synthesize RNA after keratin synthesis begins.

INTRODUCTION

The embryonic chick feather develops as an outgrowth from the embryonic epidermis. By about 12 days of incubation the feather primordia reach a form approximating the definitive organ (Waterson, 1942). Keratin then becomes detectable by histological criteria and X-ray diffraction (Bell and Thathachari, 1963) and by electron microscopy (Matulionis, 1970). The synthesis of keratin begins at the tip of the sheath and spreads downward and inward through the various feather cell types (Matulionis, 1970; Bell and Merrill, 1967). The cells fill with keratin and eventually die.

Few studies on the kinetics and control of keratin synthesis have been conducted. Bell (1964) studied the effect of actinomycin D on protein synthesis in feathers of various ages, and concluded that keratin was synthesized on stable mRNA (messenger ribonucleic acid). Yatvin (1966 *a, b*) presented evidence that pituitary hormones were required for the onset of keratin synthesis, as judged by changes in polysome profiles. Kischer and Furlong (1967) found that DNA polymerase

activity decreased rapidly at about the time of the onset of keratin synthesis, suggesting that DNA synthesis and keratin synthesis were mutually exclusive events. Malt and Bell (1965) and Ben-Or and Bell (1965) studied the development of feather proteins by chemical and immunological methods, but did not definitively identify any keratin proteins.

Harrap and Woods (1964 *a, b*; 1967) and Woods (1971) prepared soluble *S*-carboxymethyl proteins from adult feathers. These were all of similar molecular weight (~10,500), but were electrophoretically and chromatographically distinguishable. Kemp and Rogers (1972) investigated the properties of *S*-carboxymethyl proteins from newly hatched embryonic and adult feather and scale proteins. Several analytical methods for fractionation of the proteins were described, and it was concluded that a large number of very similar keratin proteins were present.

Feather keratin has been shown by electron microscopy of the fully keratinized tissue to exist

as filaments, approximately 30 Å in diameter (Rogers and Filshie, 1963). X-ray diffraction studies indicated that these consist of highly ordered aggregates of the monomers of molecular weight 10,500 (Fraser et al., 1971; Burke, 1969). The fibrils could be detected in the developing embryonic feather cells by about 13 days (Matulionis, 1970).

In the present paper, the synthesis of feather keratin proteins during embryonic development was studied by quantitative gel electrophoresis of reduced and carboxymethylated protein preparations. The results demonstrated a coordinated synthesis of the keratin proteins, as opposed to the two-stage model of Malt and Bell (1965). The results from gel electrophoresis correlated well with electron microscope visualization of feather keratin fibrils, providing a method for studying the relationship of DNA, RNA, and keratin synthesis within single cells.

MATERIALS AND METHODS

Chick Embryos

Fertilized white-leghorn eggs, a mixture of strains Para 1, 2, and 3, were obtained from Parafield Poultry Station, Parafield, South Australia. The eggs were stored at 10°C for no longer than 7 days, and incubated at 37.8°C, 54% humidity in a forced-draught incubator (Saunders Products Pty. Ltd., Adelaide, South Australia) for the required time. Feathers from eggs which had been incubated a total of, for example, 14 days were designated "14-day feathers."

Preparation of Embryonic Feathers

Embryos were carefully removed from the eggs and rinsed twice with Hanks' solution. Body feathers free from skin were plucked using jewellers' forceps and placed in Hanks' solution. When measurement of the packed volumes of feathers was required, the feathers were placed in graduated glass centrifuge tubes in Hanks' solution and centrifuged at 4,500 *g* for 10 min, using an MSE Super Minor Centrifuge (Measuring and Scientific Equipment Ltd., London, England). The packed volumes were measured immediately after centrifugation. The volumes of feathers used ranged from 0.1 to 0.5 ml in different experiments. All operations were performed at room temperature (~25°C).

Extraction of Feather Proteins

After determination of packed volumes, the feathers were incubated at 37°C for 1 h on a gyrorotary shaker (New Brunswick Scientific Co. Inc., New Brunswick, N. J.) in 10.0 ml of a solution containing 0.1 M β -mercapto-

ethanol, 8 M urea, and 0.5 M ethanolamine-HCl, pH 10.5. They were then dispersed in a hand-held Potter-Elvehjem homogenizer, and incubated for a further 2 h. 6.0 ml of a solution containing 3.0 M Tris-HCl, pH 8.0, was then added, and the proteins were carboxymethylated by the addition of 2.0 ml of 30% wt/vol solution of iodoacetic acid in 0.3 M Tris, adjusted to pH 8.2 with KOH. Final pH of this reaction mixture was 8.7. After standing for 20 min at room temperature, 0.3 ml of β -mercaptoethanol was added and the mixture was incubated at 37°C for 30 min. 2.0 ml of the same iodoacetate solution was added, followed after 20 min at room temperature by 0.4 ml of β -mercaptoethanol. Insoluble material was removed by centrifugation at 36,000 *g* for 1 h at 25°C in an MSE 1800 centrifuge. The supernate was dialysed exhaustively against bidistilled water.

During dialysis, varying amounts of a stringy, white precipitate formed. This was particularly marked in extracts from younger tissue and increased with increasing times of dialysis. Removal of the precipitate by centrifugation resulted in the selective depletion of several of the bands of intermediate mobility on pH 9.5 polyacrylamide gels (see Results). The precipitate was therefore evenly dispersed by homogenization immediately before sampling for the determination of protein content. The homogenized dialysates were made up to a known volume with water, and the protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard. Values were expressed as milligram of protein per milliliter of packed feather tissue. The preparations were freeze dried, and taken up at a protein concentration of 10 mg/ml in 4 M urea.

The extracts, particularly from the younger tissue, were contaminated with nucleic acids, as determined by UV spectra. No attempt was made to remove the nucleic acids as these do not interfere appreciably with the Lowry reaction (Lowry et al., 1951) and do not stain with Coomassie brilliant blue (Dahlberg et al., 1969).

Quantitative Polyacrylamide

Gel Electrophoresis

Samples containing known amounts of protein were fractionated by polyacrylamide gel electrophoresis at pH 9.5, 7.5, or 2.7 as described previously (Kemp and Rogers, 1972). In any one experiment, identical amounts of total protein from each embryonic feather extract were applied to the gels; usually the amount was either 50 or 100 μ g of protein. A standard curve was obtained in each experiment by running a set of gels loaded with known amounts, determined by the method of Lowry et al. (1951), of total 21-day embryonic feather proteins. After the run, protein in the gels was precipitated in 10% TCA for 30 min and then stained for 1-2 days in 0.05% Coomassie brilliant blue in 10% TCA (Chrambach et al., 1967). The gels were then rinsed in 60% ethanol to

remove precipitated dye and allowed to stand overnight in 10% TCA in the dark. The band patterns were recorded by densitometry ("Densicord," Photovolt Corp., New York). The areas under appropriate peaks of the densitometer traces were calculated, using an automatic integrator ("Integrath," Model 49, Photovolt Corp., New York).

The amount of protein in each band was determined from the standard curve, assuming equal dye binding by the proteins in each band. This assumption appears valid for the different feather keratin proteins (I. D. Walker, personal communication).

Incorporation of [¹⁴C]Leucine by Feather Tissue

12–15 day feathers were collected and washed in Hanks' solution as described above. They were then incubated for 2 h at 37°C in Hanks' solution, containing amino acids at the concentrations used in Charity Waymouth's medium, supplemented with serine (75 mg/liter) and alanine (50 mg/liter). [¹⁴C]Leucine was omitted, and 5.0 μCi/ml of [¹⁴C]leucine (sp. act. 316 mCi/mmol) was added. The medium contained chloramphenicol (10 μg/ml) and penicillin (10 μg/ml). Incubation was terminated by removing the incubation medium and adding 1.0 ml of cold Hanks' solution containing 0.1% leucine. The packed volumes of the feathers were then determined, and protein was extracted as described above.

Polyacrylamide Gel Electrophoresis of Radioactive Proteins

Samples containing known amounts of [¹⁴C]protein were subjected to polyacrylamide gel electrophoresis at pH 7.5 as described above except that ethylene diacrylate was used as the cross-linking agent in the gels to facilitate the subsequent determination of radioactivity (Cain and Pitney, 1968). It should be pointed out that initial attempts were made to use the pH 9.5 system in this manner but the results were poorly reproducible because the gels were unstable. This finding contrasts with the results of Cain and Pitney (1968). Ethylene diacrylate gels were stable at pH 7.5. After electrophoresis, the gels were stained and analysed by densitometry as described above. The gels were then sliced into 1-mm segments using a razor blade gel slicer (Mickle Engineering Co., Gomshall, Surrey, England). Each slice was placed in a glass vial (0.8 cm × 4.5 cm) and solubilized by treatment with 1.0 M ammonium hydroxide (0.4 ml; 60°C for 2 h, or overnight at room temperature). The solution was absorbed onto a glass fibre filter and dried at 110°C for the determination of radioactivity.

Incorporation of [³H]Thymidine and [³H]Uridine by 12-Day Feathers

In order to prevent morphological damage which might interfere with subsequent electron microscopy,

feathers were carefully teased from 12-day embryos still attached to small pieces of skin using jeweller's forceps. As much as possible of the attached skin was then removed from each feather, taking care not to grasp the feathers themselves with the forceps during these operations. The feathers were then incubated for 30 min at 37°C in Charity Waymouth's medium containing 50 μCi/ml of [³H]thymidine (sp. act. 100 mCi/mmol) or [³H]uridine (sp. act. 31 Ci/mmol). The medium was then drained off, and the feathers were washed quickly twice with cold medium containing unlabeled uridine or thymidine (1 mg/ml) and prepared for electron microscope autoradiography. In control experiments, actinomycin-D was present during the incubation with [³H]uridine at a concentration of 50 μg/ml (Bell and Merrill, 1967).

Electron Microscopy

Samples were fixed for 1 h in a solution containing 2% vol/vol glutaraldehyde and 0.1 M sodium cacodylate, pH 7.2, and then washed in a solution containing 0.18 M sucrose in 0.1 M sodium cacodylate for 30 min. They were postfixed in a solution containing 2% wt/vol osmic acid in 0.1 M sodium cacodylate for 30 min, followed by a wash in 1% wt/vol uranyl acetate, and dehydration through graded acetones. The samples were then embedded in Araldite. Sections were cut on an LKB microtome (Watson-Victor, Pty. Ltd., Australia), using glass knives. The sections were picked up on carbon-coated grids, and stained for 15 min in lead citrate. The sections were examined in a Siemens Elmiskop I (Siemens Industries Ltd., Australia) at 80 kV, using a 50-μm objective aperture.

Electron Microscope Autoradiography

After incubation with [³H]thymidine or [³H]uridine, the feathers were fixed and stained as described above, except that the glutaraldehyde fixative contained 1 mg/ml unlabeled thymidine or uridine. After staining, the sections were coated with a thin carbon film and covered with a film of Ilford L4 photographic emulsion (Ilford Ltd., Essex, England). The sections were exposed for 60 days at 4°C, and then developed in Microdol (Caro, 1964) or fine grain developer (Pawletz, 1967).

RESULTS

Changes in Total Protein Content during Feather Development

Total protein contents of feathers at various developmental ages are shown in Fig. 1. Statistical analysis of the data (see caption to Fig. 1) demonstrated that significant increases in protein content occurred between days 13 and 14 and between days 14 and 15. By day 15 the protein content was over double that at day 13, indicating a dramatic

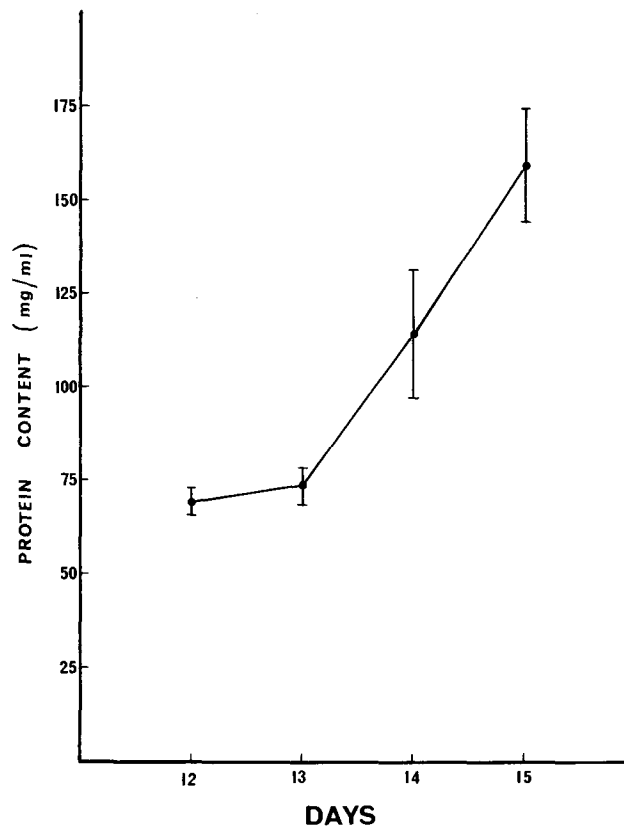


FIGURE 1 Total protein content of feathers during development. Total protein content per milliliter of packed feathers was determined as described in Materials and Methods. Results shown are the means obtained from five independent experiments, plus and minus the standard errors of the means. In two of these experiments, the tissues had previously been incubated for 2 h with [14 C]leucine, as described in Materials and Methods. Analysis of variance showed a significant effect arising from feathers of different ages ($P = 0.002$). Although there was some scatter between the independent experiments, the effect was just above the critical α -level of 0.05 ($P = 0.0538$), and application of the Newman-Keuls A-Posteriori Test Between Means (Winer, 1962) established that differences between the ten possible pairs of experiments were not significant ($\alpha = 0.05$) in all but one instance. The difference between the protein content at days 12 and 13 did not reach significance ($P > 0.05$), but significant differences between the protein content were found for days 13 and 14 ($P < 0.05$) and days 14 and 15 ($P < 0.01$).

change in rate of protein synthesis at about 13 days.

In individual experiments, values consistently higher or lower than the mean for protein content at each age were observed. Whether this effect resulted from the extraction procedure or from differences in batches of embryos is not known. However, in every experiment, the observed protein content increased between days 13 and 14 and between days 14 and 15.

The scatter in experimental points at days 14 and 15 presumably also reflects to some extent the individual variation in development of different embryos. At these ages, as the protein content is

rapidly increasing, variations in individual development over a total time of about 12 h, spread across the mean, would account for much of the observed scatter. Such variation in developmental age would be expected (Lillie, 1965).

Determination of Keratin Content during Feather Development by Quantitative Polyacrylamide Gel Electrophoresis

Aliquots of the reduced and carboxymethylated protein preparations were fractionated by polyacrylamide gel electrophoresis, stained and

analysed by quantitative densitometry as described in Materials and Methods.

After fractionation by polyacrylamide gel electrophoresis at pH 9.5, marked differences in patterns were observed between protein preparations depending on whether the precipitate formed during dialysis was removed by centrifugation or not. The γ -proteins (Kemp and Rogers, 1972) were removed to a varying extent by this step, as was found for 21-day embryonic feather proteins (Kemp, 1972). In order to avoid removing some of the slower moving β -components as well (Kemp, 1972), centrifugation at this stage was omitted in subsequent studies.

In the 11- and 12-day feather extracts, protein bands corresponding in mobility to the major feather keratins (bands $\beta 2$ - $\beta 5$; Kemp and Rogers, 1972) were present only as traces (Fig. 2). These bands rapidly increased in quantity after day 12, and had already become the most abundant protein species in the feathers by day 15.

The α -proteins appeared to follow a similar course during development to the β -proteins, but were not detectable as early, presumably because of their low amount. The γ -proteins followed a different pattern during development. These were readily detectable at 11-12 days, and did not undergo a similar rapid increase in amount after day 12.

The precise pattern of protein bands in the γ -region varied somewhat in different experiments. However, their pattern was very similar from gel to gel in each individual experiment, and the conclusions above could always be drawn.

The patterns of keratin bands obtained by polyacrylamide gel electrophoresis at pH 7.5 (Fig. 3) were essentially identical to those obtained by polyacrylamide gel electrophoresis at pH 9.5 (Fig. 2), in that the β -bands rapidly increased in amount after day 12. The pattern in the γ -region was somewhat different, however. The value of this system lay in its applicability to the fractionation of radioactive proteins (see below).

Fractionation of proteins from 11 to 21 day feathers by polyacrylamide gel electrophoresis at pH 2.7 is shown in Fig. 4.

After electrophoresis in this system the major keratin bands A-H (Kemp and Rogers, 1972) were not as well resolved from the background of nonkeratin proteins as in the high pH system. It was clear, however, that there was a rapid increase in amount of each keratin band after day 12.

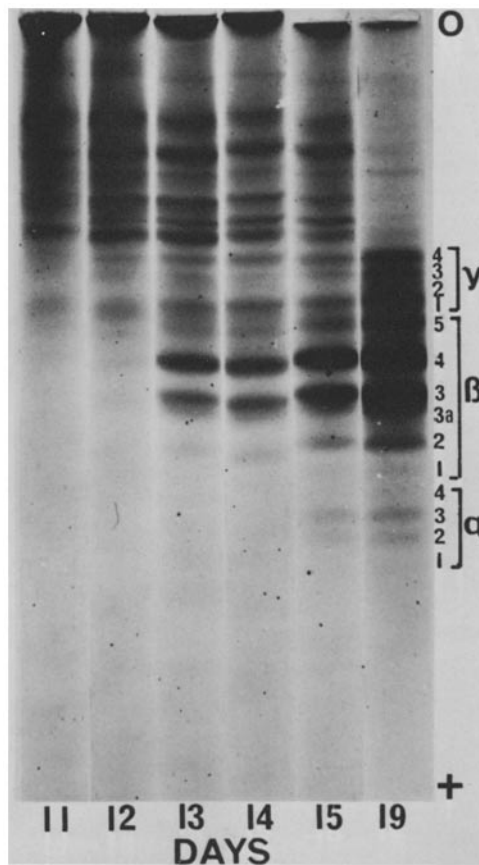


FIGURE 2 Polyacrylamide gel electrophoresis at pH 9.5 of proteins from feathers during development. Samples (100 $\mu\text{g/gel}$) of the reduced and carboxymethylated protein preparations from feathers at 11-19 days of development were subjected to polyacrylamide gel electrophoresis at pH 9.5 and stained with Coomassie blue as described in Materials and Methods. O, origin; +, anode. Bands $\alpha 1$ - $\gamma 4$ are indicated.

The amounts of the major keratin bands resolved in each of the three polyacrylamide gel electrophoresis systems per milliliter of packed 12-15 day feathers are shown in Figs. 5-7. Similar results were obtained in all three systems. An increase in amount of each band occurred between days 12 and 13, followed by a more rapid increase during each of the next 2 days. Furthermore, the amounts of each band relative to the others remained approximately constant at each age.

Examination of photographs of pH 9.5 and pH 7.5 gels (Figs. 2 and 3) reveals a background smear of stained material, predominantly of lower mobility than band $\beta 4$, which decreased as both develop-

mental age and band mobility increased. The relatively high values obtained at days 12 and 13 for band β_5 , would appear to derive from this background. It is also evident that all values obtained at day 12 would include some contribution from background staining. Quantitation of the γ -bands was not possible, as the background was too high in the region.

Changes in Total Keratin Content during Feather Development

Estimations of total keratin content were made (Fig. 8) by summing the values for each keratin

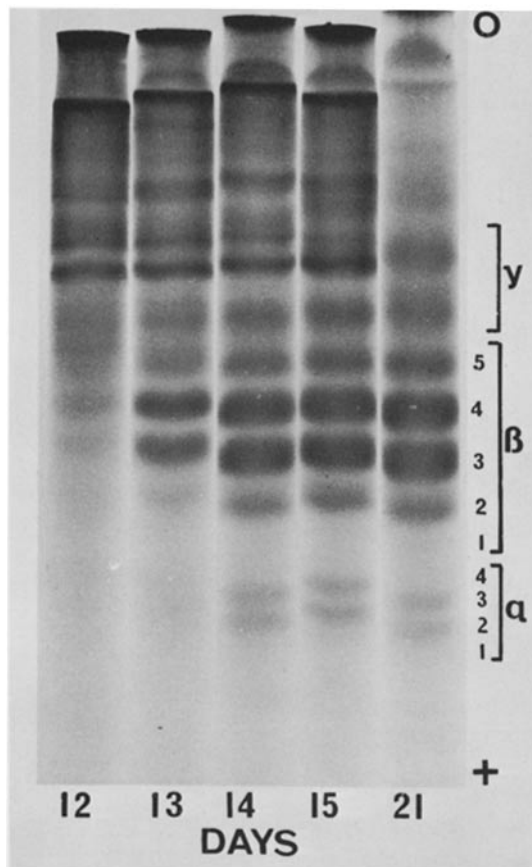


FIGURE 3 Polyacrylamide gel electrophoresis at pH 7.5 of proteins from feathers during development. Samples (100 $\mu\text{g/gel}$) of the reduced and carboxymethylated protein preparations from feathers at 12–15 days of development were subjected to polyacrylamide gel electrophoresis at pH 7.5. For comparison, the pattern at 21 days (newly hatched chick) is also shown. o, origin; +, anode. Bands α_1 – β_5 and the γ -bands are indicated.

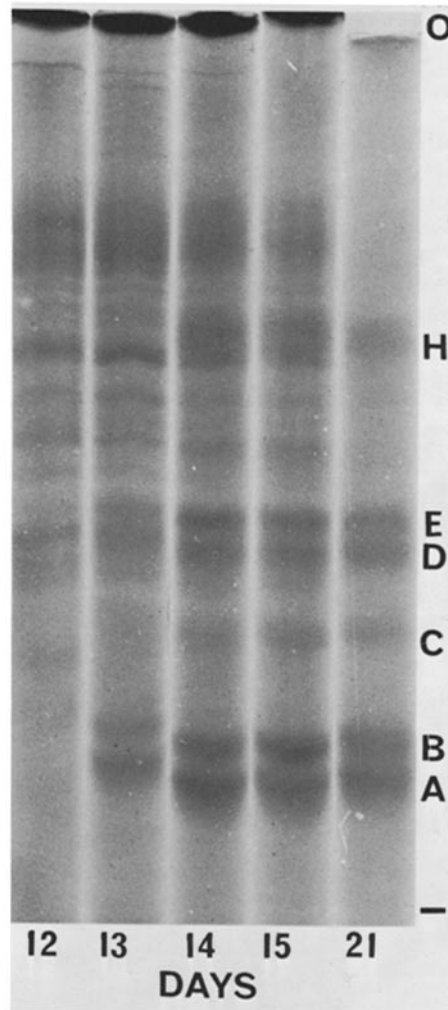


FIGURE 4 Polyacrylamide gel electrophoresis at pH 2.7 of proteins from feathers during development. Samples (50 $\mu\text{g/gel}$) of the reduced and carboxymethylated protein preparations from feathers at 12–15 days of development were subjected to polyacrylamide gel electrophoresis at pH 2.7 for 6 h at 2 mA/gel. For comparison the pattern obtained from 21-day feather protein is shown. O, origin; -, cathode.

band resolved by polyacrylamide gel electrophoresis at pH 9.5, 7.5, or 2.7 (Figs. 5–7) of the proteins in preparations from feathers of each age. The values obtained from the three different gel systems were in good agreement. Furthermore, the increase in total keratin content between days 13 and 14 was 102% and that between days 14 and 15 was 69% of the increase in total protein content

between these days, respectively, indicating that keratins were the major proteins synthesised.

Determination of the Rates of Keratin Synthesis during Feather Development by Incorporation of [¹⁴C]Leucine

Studies on the incorporation of [¹⁴C]leucine into acid precipitable material using 14-day feathers gave a linear rate of incorporation over a 2-h period and indicated that the tissue had remained viable. Plucked feathers from 12- to 15-day embryos were therefore incubated with [¹⁴C]leucine for 2 h and the radioactive proteins extracted. The

total incorporation per milliliter of packed feathers at each age (Table I) varied somewhat in the different experiments.

Samples of the radioactive proteins were fractionated by polyacrylamide gel electrophoresis at pH 7.5. Densitometer tracings and radioactivity profiles of such gels are shown in Fig. 9. The profiles were very similar in the different experiments. In particular, very little radioactivity was associated with the keratin region of the gel at day 12, whereas by days 14 and 15, keratin proteins were the predominant radioactive species.

Keratin synthesis at each age was estimated by summing the radioactivity of gel slices in each

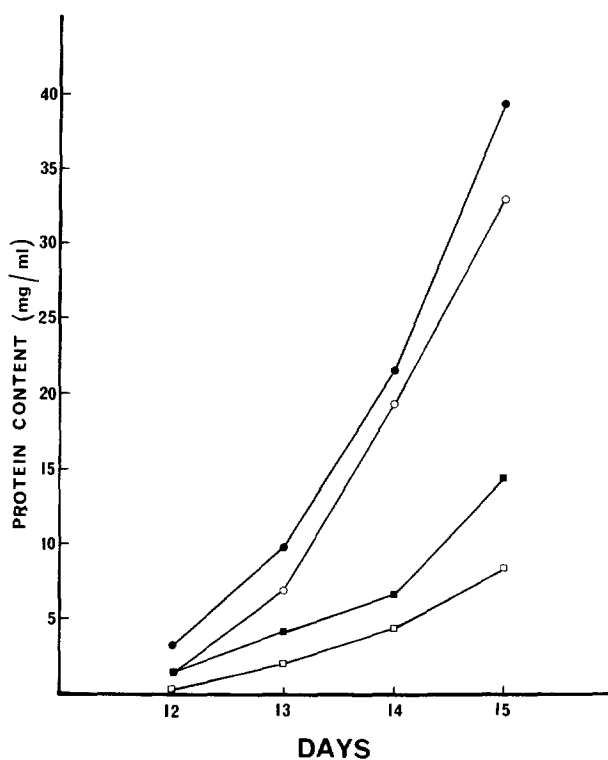


FIGURE 5 Changes in keratin content during feather development as determined by polyacrylamide gel electrophoresis at pH 9.5. Values shown for bands β_2 - β_5 are the average of two independent experiments, as in Fig. 2. Gels were run in duplicate in each experiment. Values are expressed as milligrams of protein in each resolvable band per milliliter of packed feather tissue. As the peaks were not completely resolved by densitometry after polyacrylamide gel electrophoresis at pH 9.5 and pH 7.5, the number of integrator units between the lowest point of successive "troughs" in the densitometer traces was considered to represent the area of the peak between them. Although this procedure would presumably result in the overestimation of some peaks and underestimation of others, the similarity in profiles of the keratin-containing regions of the gels at days 13-15 should ensure that such weighting of peaks is relatively constant at each age, thereby providing a valid means of comparison. ●—●, band β_4 ; ○—○, band $\beta_{3a} + \beta_3$; ■—■, band β_5 ; □—□, band β_2 .

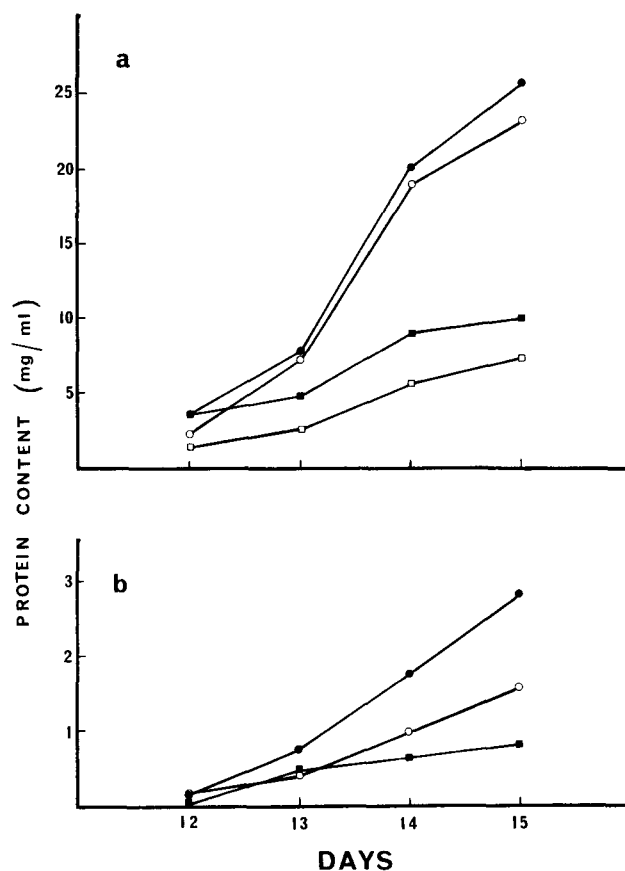


FIGURE 6 Changes in keratin content during feather development as determined by polyacrylamide gel electrophoresis at pH 7.5. (a) Values shown for bands $\beta 2$ – $\beta 5$ are the average of two independent experiments, as in Fig. 3. Other details as for Fig. 5. (b) Values shown for bands $\alpha 1$ – $\alpha 4$ are from one experiment only, as in Fig. 3, but the gels were overloaded ($500 \mu\text{g/gel}$) to allow densitometric quantitation of the α -bands. ●—●, bands $\alpha 2$ and $\alpha 3$; ○—○, band $\alpha 4$; ■—■, band $\alpha 1$.

band of the keratin-containing region of each gel (Fig. 10). As estimated in this manner, keratin synthesis in incubated feather tissue accounted for only 7% of the total protein synthesis in 12-day feathers and had increased to greater than 25% of total protein synthesis in 14- and 15-day feathers (Fig. 10, a). This increase could be accounted for by an increase in the rate of keratin synthesis in tissue at these ages (Fig. 10, b).

The rates of synthesis of keratin proteins (polyacrylamide gel bands $\beta 2$ – $\beta 5$) were approximately constant relative to each other at days 13 to 15, within the accuracy of the determination (Fig. 10, c). The relatively high value obtained at day 12 for bands $\beta 4$ and $\beta 5$ would appear to result from the low radioactivity and contamination of this area of

the gel with a background smear of nonkeratin proteins as can be seen in Fig. 9. The results are therefore in agreement with the results obtained by quantitative densitometry (Fig. 6). Moreover, the decrease of leucine incorporation into keratin between days 14 and 15 reflects the decreased average incorporation of leucine at day 15 relative to day 14 (Table I).

Electron Microscope Observations on Keratin Synthesis during Feather Development

Electron micrographs of transverse sections of feathers at various ages of development are shown in Figs. 11–14. Relatively few keratin fibrils were

detectable in 12-day feathers, except in the sheath cells, in agreement with the results of Matulionis (1970). The content of keratin fibrils was greater at the tip of the feather than at the lower level shown in Fig. 12, and there was some variation in feathers from different 12-day embryos. At this age, keratin fibrils are not obvious at the magnifications shown although they are clearly seen at higher magnification (see Fig. 15). This is in marked contrast to the situation at later stages. After day 12, the fibrils rapidly increase in abundance until by day 15, fibrils account for the majority of the cytoplasmic area. The quantitative observations on synthesis of feather keratin proteins described above therefore correlate well with electron microscope visualization of keratin fibrils during development of the feather.

$[^3\text{H}]$ Thymidine Autoradiography

Initially it was shown by light microscope autoradiography that in 12-day feathers labeled with a 30-min pulse of $[^3\text{H}]$ thymidine, many nuclei

of barb and barbule cells in the proximal one-third ("basal region") of the feathers were labeled. The nuclei of these cells in the distal two-thirds of the feather were not labeled. Areas from both basal and tip regions were subjected to close examination by electron microscope autoradiography (Fig. 15). Although barb and barbule cells at the tip region contained keratin fibrils (Fig. 15 *a*), no cells which had incorporated thymidine were found to contain keratin fibrils. The cells examined in this manner are described by Table II.

From these observations it was concluded that keratin fibrils were not detectable in cells which had incorporated thymidine for 30 min before examination. They were present only in cells that were amongst the population without labeled nuclei.

$[^3\text{H}]$ Uridine Autoradiography

In contrast to the results from $[^3\text{H}]$ thymidine autoradiography, cells were found which had actively incorporated $[^3\text{H}]$ uridine into both nucleus

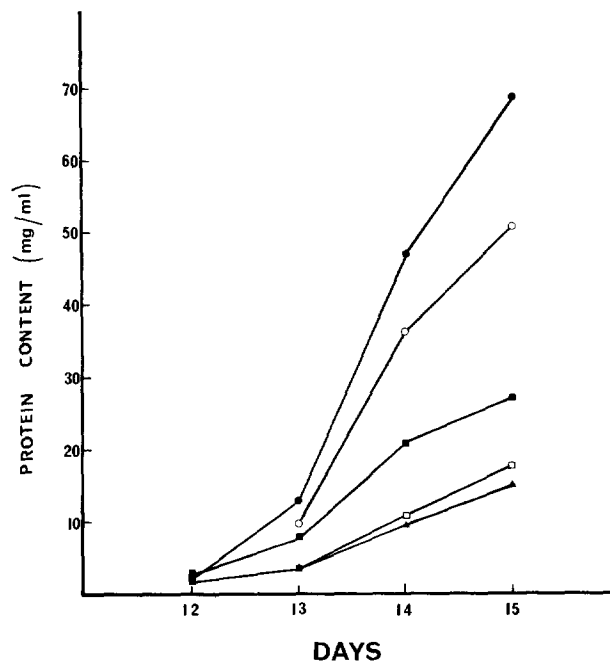


FIGURE 7 Changes in keratin content during feather development, as determined by polyacrylamide gel electrophoresis at pH 2.7. Values for bands A + B, C, and D + E are the average of two independent experiments, as in Fig. 4. Gels were run in duplicate in each experiment. Values are expressed as milligrams of protein in each band (or group of bands) per milliliter of packed feather tissue. The individual bands in the groups A + B, D + E were not resolved sufficiently for their individual quantitation, and as a consequence, they were quantitated as these groups. The curve shapes and relative areas of bands A and B, however, were determined by least squares analysis of the densitometer traces (R. D. B. Fraser and E. Suzucki, personal communication). ●—●, bands A + B; ○—○, band A; ■—■, bands D + E; □—□, band B; ▲—▲, band C.

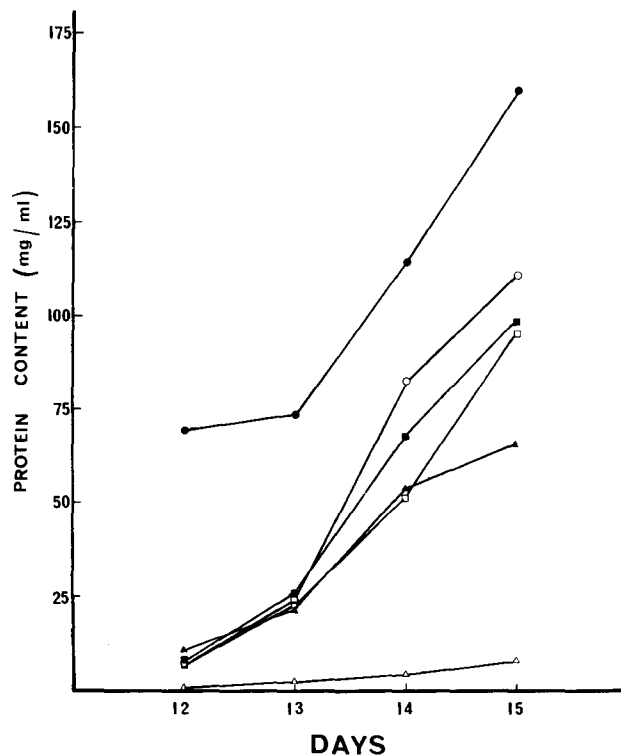


FIGURE 8 Changes in total keratin content during feather development. The values shown were obtained by summing the values for keratin bands at each age in Figures 5, 6, or 7. ●—●: Total protein content (from Fig. 1). □—□: Total content of bands $\beta 2-\beta 5$ determined by polyacrylamide gel electrophoresis at pH 9.5. ▲—▲: Total content of bands $\beta 2-\beta 5$ determined by polyacrylamide gel electrophoresis at pH 7.5. ○—○: Total content of bands A-E determined by polyacrylamide gel electrophoresis at pH 2.7. △—△: α -band content (from Fig. 6, b). ■—■: Total keratin content. The mean of the values obtained for the sum of bands $\beta 1-\beta 5$, as determined by polyacrylamide gel electrophoresis at pH 9.5 and pH 7.5, and bands A-E as determined by polyacrylamide gel electrophoresis at pH 2.7 was determined. The value obtained for the α -bands (Fig. 6, b) was then added to the mean. As the α -bands are thought to account for the H bands at pH 2.7 (I. D. Walker, personal communication), this procedure should give the total value for all keratin components except any which run in the γ -region of pH 9.5 and 7.5 gels.

TABLE I
Incorporation of [^{14}C]Leucine Per Milliliter of
Feathers in Culture at Days 12-15

Day	12	13	14	15
Experiment 1	5.8	3.9	5.2	6.2
Experiment 2	8.9	7.7	6.9	3.3
Average	7.3	5.8	6.0	4.7

Feathers of each age were incubated with L-[^{14}C]leucine. The tissue volumes, protein content, and radioactivity were determined as described in the Materials and Methods section. Values are expressed as counts per minute per milliliter tissue per hour $\times 10^{-5}$.

and cytoplasm and also contained keratin fibrils (Fig. 16). The incorporation of [^3H]uridine was reduced by 70% by the presence of actinomycin D during the incubation as determined by autoradiography at the light microscope level, indicating that the [^3H]uridine had been incorporated into RNA. It was concluded that cells containing keratin fibrils are capable of RNA synthesis. The nature of this RNA was not investigated.

It was observed that a large percentage of the silver grains derived from the labeled RNA was localized in the immediate vicinity of keratin fibrils. A similar localization of labeled uridine

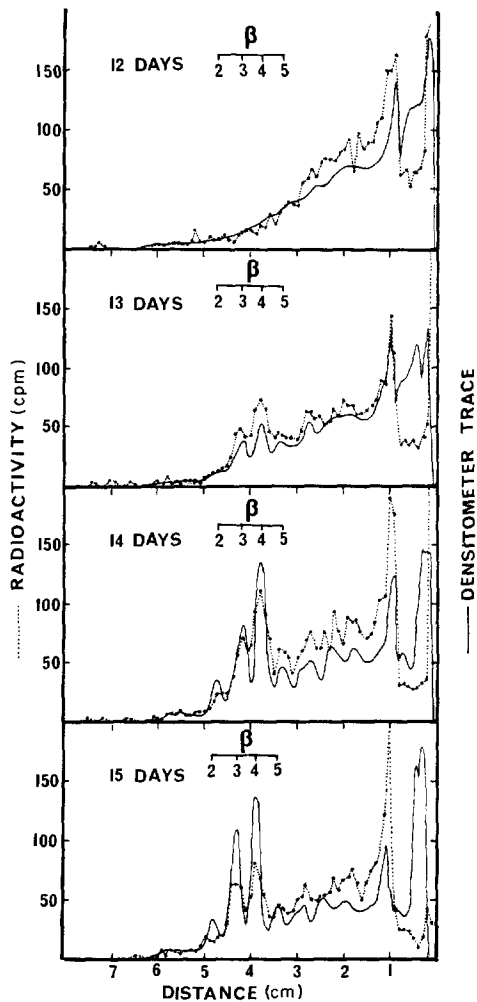


FIGURE 9 Polyacrylamide gel electrophoresis at pH 7.5 of proteins labeled with [^{14}C]leucine. Samples (400 $\mu\text{g/gel}$) of reduced and carboxymethylated proteins from feathers at 12–15 days of development, which had been labeled for 2 h with [^{14}C]leucine, were subjected to polyacrylamide gel electrophoresis at pH 7.5 as described in Materials and Methods. The gels were crosslinked with ethylenediacrylate (Cain and Pitney, 1968). The results shown are from experiment one of Table I. Bands β_2 – β_5 are indicated.

with developing keratin fibrils was observed in the guinea pig hair follicle and it was suggested that keratin synthesis occurs at the surface of the fibrils (Rogers, 1969, Fraser et al., 1972).

DISCUSSION

Changes in Protein Content and Synthesis

The techniques described for measuring both the total protein content and the quantity of each

keratin protein band resolved on polyacrylamide gels per milliliter of packed feather tissue were found to provide simple and useful methods for studying the synthesis of keratin proteins during

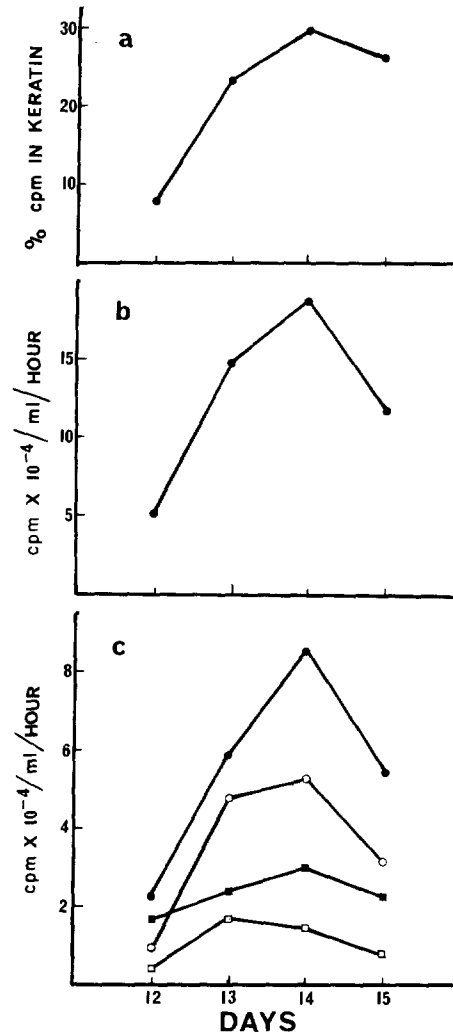


FIGURE 10 Determination of the rates of keratin synthesis during feather development by incorporation of [^{14}C]leucine. Values were obtained by summing the radioactivity of gel slices (Fig. 9) corresponding to the relevant keratin bands, and are the average of two independent experiments. (a) Incorporation of [^{14}C]leucine into keratin bands β_2 – β_5 at days 12–15, as percent of total incorporation. (b) Total incorporation of [^{14}C]leucine into keratin bands β_2 – β_5 at days 12–15, per milliliter of packed feathers, per hour. (c) Relative rates of incorporation of [^{14}C]leucine into keratin bands β_2 – β_5 at days 12–15, per milliliter of packed feathers, per hour. ●—●, band β_4 ; ○—○, bands $\beta_{3a} + \beta_3$; ■—■, band β_5 ; □—□, band β_2 .

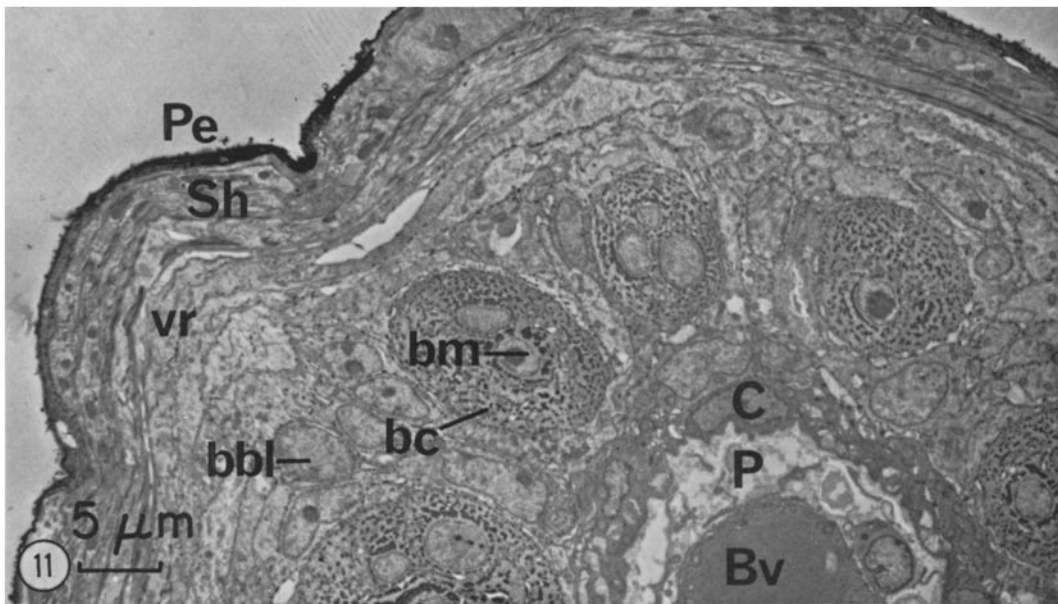


FIGURE 11 Electron micrograph showing approximately half of the cross section of a 13-day embryonic feather (feather-germ) made at a level about 5 mm from the base. A variety of differentiated cells can be seen. The cylindrical-shaped feather is segmented into barb ridges each of which is separated from the pulp cavity (*P*) by a layer of cylinder cells (*C*). In the cavity can be seen a blood vessel (*Bv*). In the centre of each barb ridge are barb cells which are of two kinds, barb medulla cells (*bm*) and barb cortical cells (*bc*). Barbule cells (*bbl*) and another group of cells, barb vane ridge cells (*vr*), are present next to the barb cells. A cylindrical layer of cells, the sheath (*Sh*), surrounds the total array of barb ridges and external to this is the periderm (*Pe*). $\times 2,130$

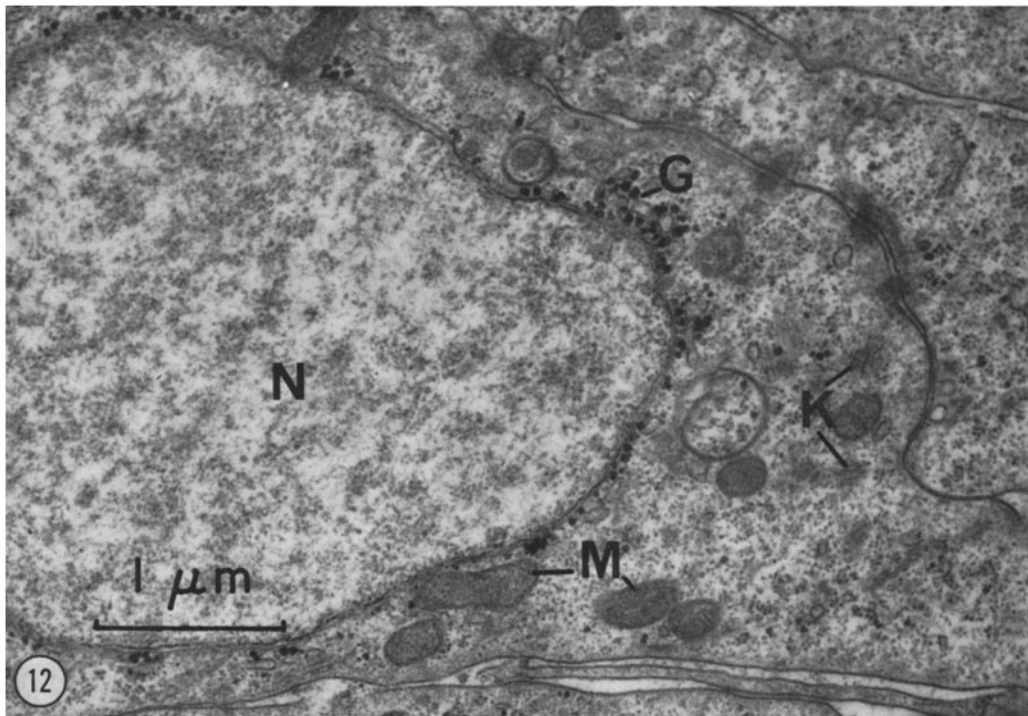


FIGURE 12 Electron micrograph of a transverse section through a cell of a barb ridge of a 12-day feather. The region of the barb ridge was about 1 mm from the base of the feather (total feather length approximately 3 mm). The nucleus (*N*) of the cell occupies a volume that is large relative to that of the cytoplasm. Possible traces of keratin (*K*) are detectable in the cytoplasm, together with small mitochondria (*M*) and glycogen particles (*G*). $\times 25,500$.

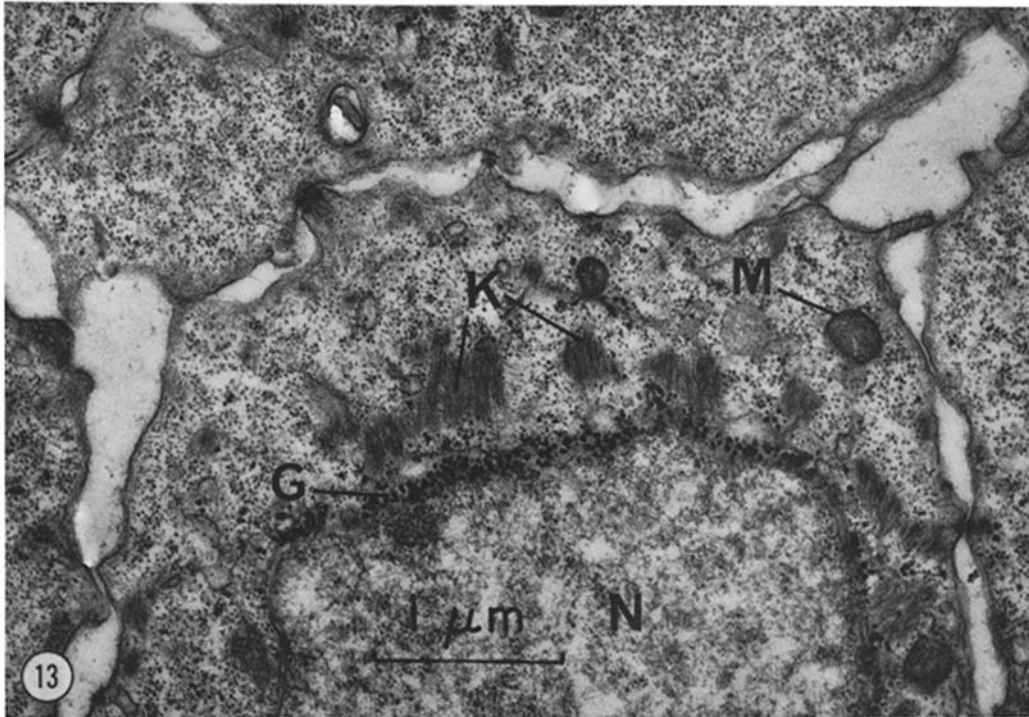


FIGURE 13 Electron micrograph of a region similar to that of Fig. 12 but of a 13-day feather (total feather length approximately 7 mm). The appearance of the portion of the barb ridge cell shown is similar to that of the earlier age except for an increase in the amount of keratin present in the cytoplasm. The mitochondria and glycogen are still present. $\times 25,500$.

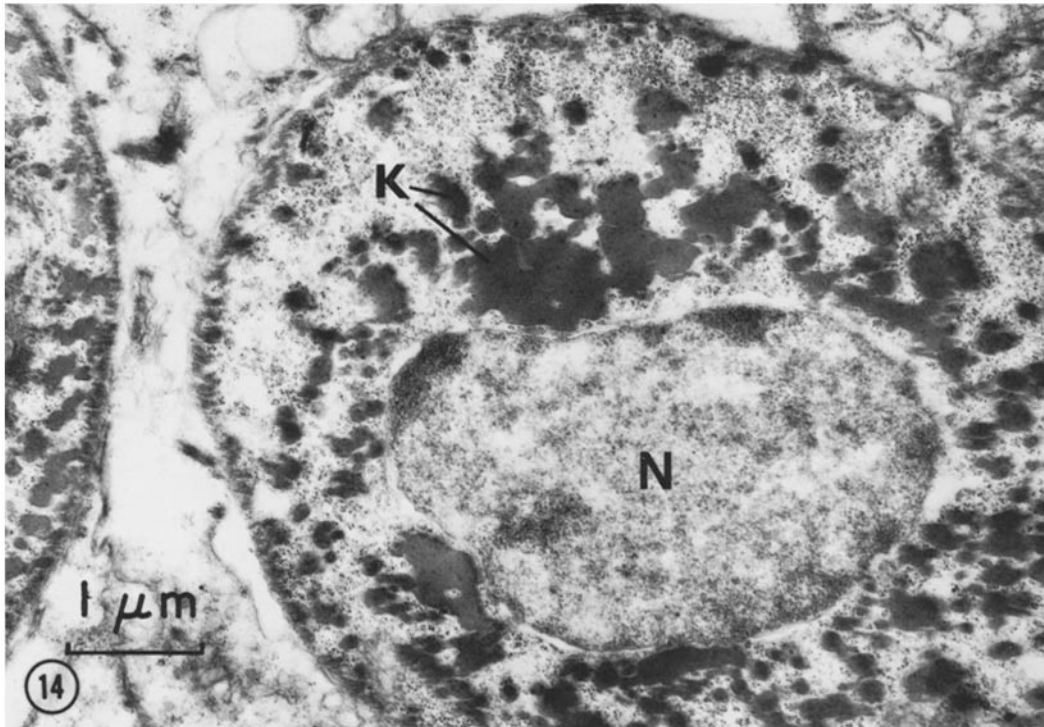


FIGURE 14 Electron micrograph of a barb ridge cell of a 15-day feather. The region as in Figs. 12 and 13 was 1 mm from the base of the feather (total feather length approximately 10 mm) and now reveals a marked increase in the quantity of keratin. The aligned fibrils are seen in cross section as dense aggregates and constitute a large fraction of the cytoplasm. The nuclear area is now relatively smaller than the cytoplasm. At this stage of keratin synthesis the glycogen particles have disappeared and the mitochondrial population has greatly decreased. $\times 18,000$.

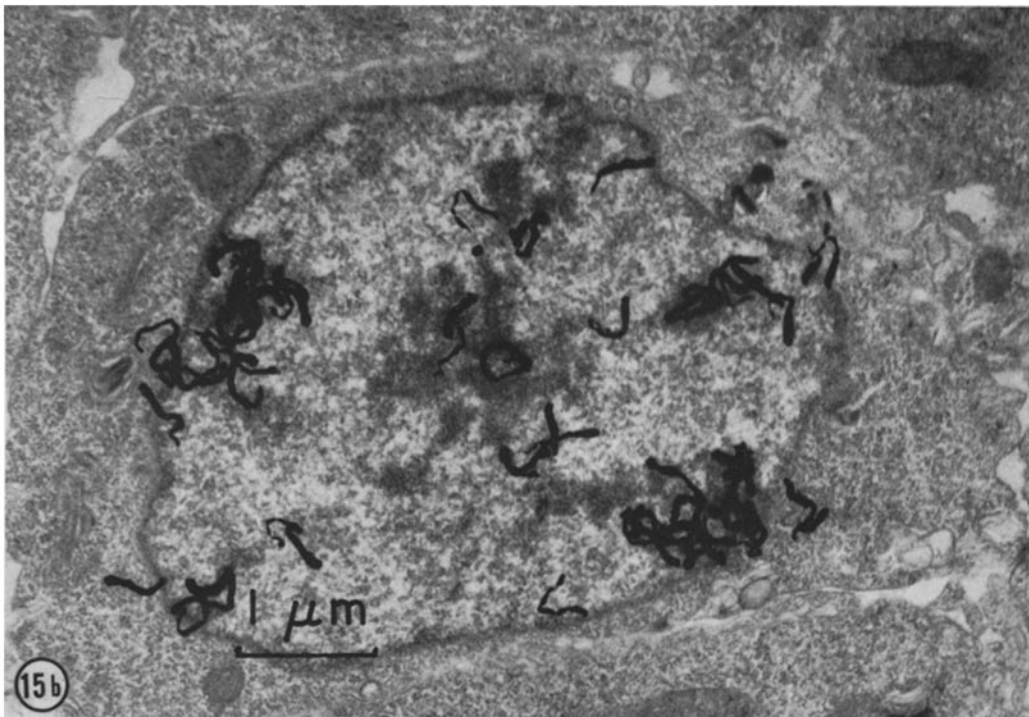
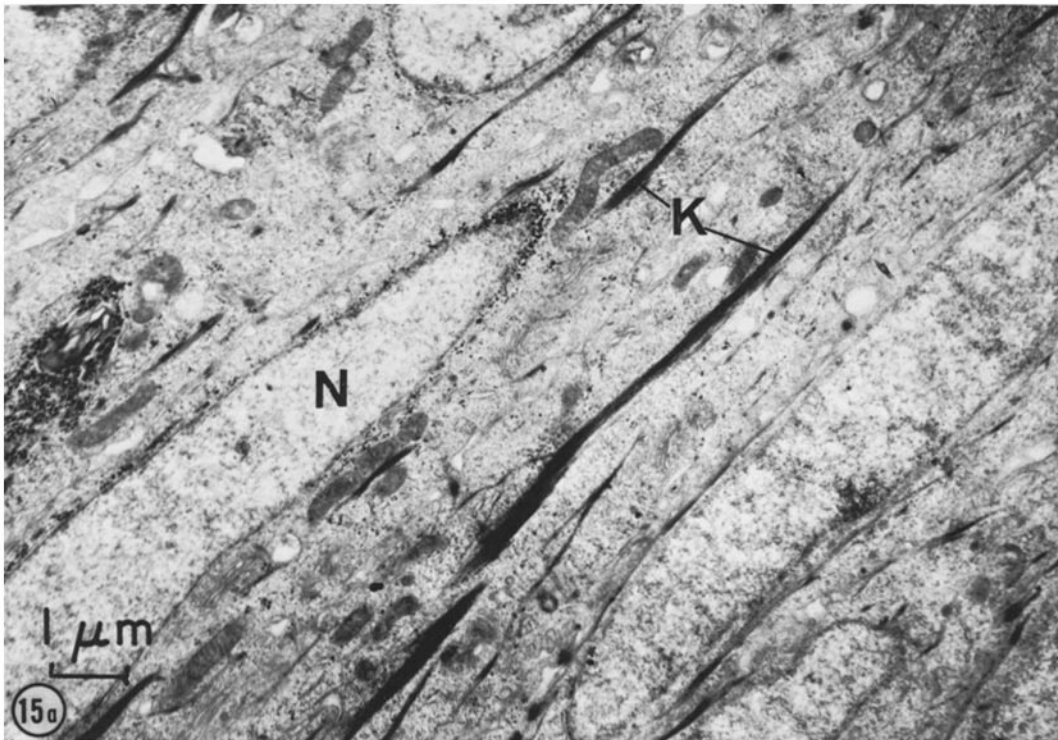


FIGURE 15 Electron micrographs of an autoradiograph of a 12-day feather labeled with [^3H]thymidine. In (a) the barbule cells at the tip of the feather, which are not labeled, contain keratin fibrils (*K*), whereas in (b) the label is restricted to the nuclei of the cells at the base of the feather which do not contain detectable keratin fibrils when examined at a higher magnification than (a). (a), $\times 9,200$; (b), $\times 18,750$.

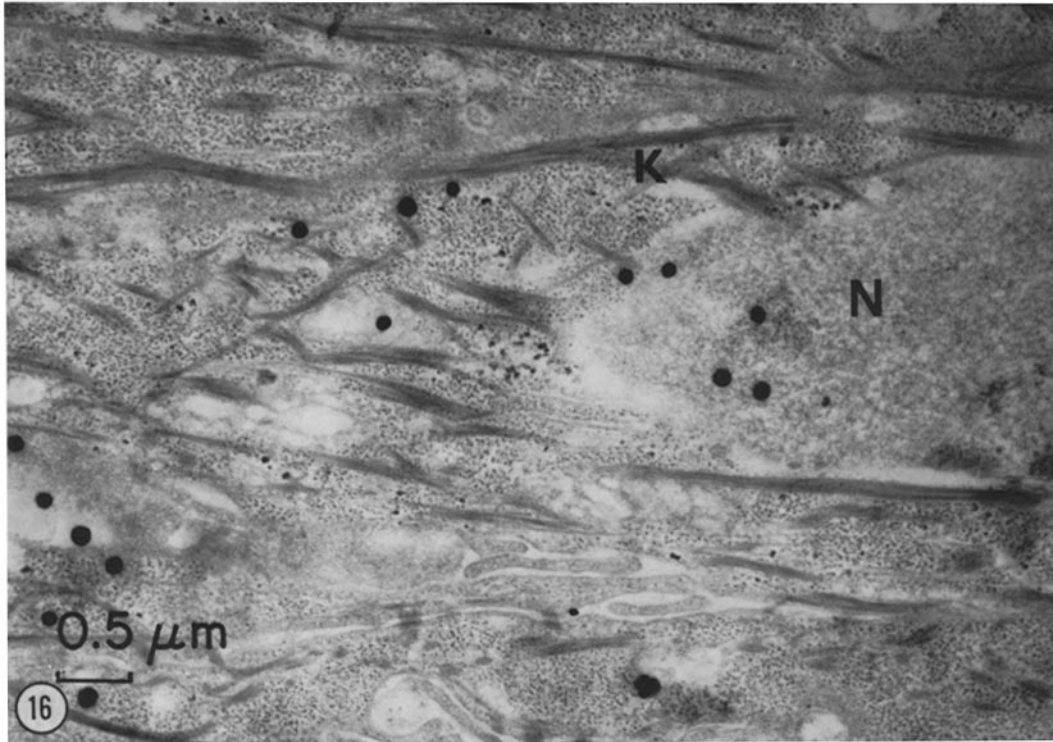


FIGURE 16 Electron micrograph of an autoradiograph of a 12-day feather labeled with [³H]uridine. This micrograph is of a portion of a barb cell from near the tip of the feather that has been sectioned so that the keratin fibrils (*K*) are shown in longitudinal aspect. The location of the silver grains over the nucleus and cytoplasm indicates labeling of the cell with uridine. $\times 18,400$.

TABLE II
Distribution of [³H]Thymidine-Labeled Nuclei
among 12-Day Barb and Barbule Cells Examined in
Detail by Electron Microscopy

Total barb cells*	185
Cells with labeled nucleus†	61
Cells containing both keratin and labeled nucleus	0
Cells containing detectable keratin	27
Average number of grains/labeled nucleus	30

* Includes the precursors of all cell types of the barb ridges (Matulionis, 1970).

† Background (number of grains over unlabeled cells) was 1.0 silver grains/cell. Cells with 2 or less silver grains/nucleus were counted as unlabeled cells.

development of the embryonic chick feather. Use of the parameter "packed tissue volume" is not ideal since the results do not necessarily reflect precisely the changes in protein content per cell.

The developing feather is a heterogeneous mix-

ture of cells. The bulk of these are of the barb and barbule type, but they change greatly in size and shape as development proceeds. It is therefore impossible at present to determine accurately the number of cells in any feather sample. Consequently, even if DNA determinations were used as the basis to express protein content, it could not be demonstrated that the results precisely reflect the change in protein content per cell. Further, it is likely that DNA is degraded and removed during later stages of cytodifferentiation as in other keratinizing tissues (Fukuyama and Bernstein, 1961; Downes et al., 1966 *a*), although no information on this question is available for the developing feather.

The total protein content per unit volume of packed feathers increased markedly between days 13 and 14 and thereafter (Fig. 1), suggesting a rapid change in the net rate of protein synthesis. That this change principally involved the synthesis of keratin proteins was demonstrated quantitatively by gel electrophoresis (Figs. 5-7) in agree-

ment with histological and X-ray diffraction observations (Bell and Thathachari, 1963), electron microscope observations (Matulionis, 1970), and chemical and immunological observations (Malt and Bell, 1965; Ben-Or and Bell, 1965).

The incorporation of [^{14}C]leucine into the keratin proteins during this period of development further demonstrated the change in spectrum of proteins synthesized (Figs. 9 and 10). The results, in general, were in good agreement with the results from quantitative polyacrylamide gel electrophoresis. However, keratin synthesis accounted for a maximum of only 30% of total protein synthesis when determined by the radiochemical technique. This result would not appear to be compatible with the observation (Fig. 8) that keratin synthesis accounted predominantly for the increase in protein content during development. Several explanations of the discrepancy are possible. For example, the culture conditions may have been suboptimal for keratin synthesis. Alternatively, for example, the leucine pool-size may be significantly lower in basal cells than in the developmentally more advanced apical cells, or the permeabilities of the cells may vary.

Kinetics of Keratin Synthesis

Quantitative polyacrylamide gel electrophoresis (Figs. 5-7) demonstrated that within the present limits of resolution and accuracy, an increase in the amount of each of the predominant keratin protein bands began after 12 days. Furthermore, the relative amounts of these bands remained constant during development. The rates of synthesis of each major resolvable keratin band became maximal after 13 days. The α -proteins appeared to follow a similar course of development to the β -proteins, but were not measurable as early because of their relatively low amount. In contrast, the γ -proteins were detectable at 11 days, and did not undergo the same increase after day 12.

Malt and Bell (1965) postulated a two-phase synthesis of keratin during development of the embryonic chick feather. They suggested that a fibrous protein of low sulphur content was first synthesized, followed by the synthesis of sulphur-rich matrix proteins. However, these proteins were not definitively identified. In the case of hair and wool proteins which are known to have this microfibril-matrix structure (see Fraser et al., 1972), the available evidence supports a mode of synthesis in which the low-sulphur fibrous proteins

begin to be laid down before the matrix proteins (Downes et al., 1963; 1966 *b*; Fraser, 1969 *a,b*). Evidence from protein-chemical studies (Harrap and Woods, 1964 *a,b*; Woods, 1971; Kemp and Rogers, 1972) indicated that no such microfibril-matrix structure exists in feather keratin. Furthermore, X-ray diffraction studies (Fraser et al., 1971; Burke, 1969) support the concept that subunits of feather proteins with molecular weights of about 10,500 constitute the feather microfibrils.

Recent evidence (Kemp, 1972; O'Donnell, 1973) has conclusively demonstrated that the β -pleated sheet region of the feather keratin secondary structure consists of a polypeptide sequence of about 60 residues in the center of the keratin subunit of molecular weight about 10,500. All purified component proteins of the embryonic chick feather, denoted α and β (Kemp and Rogers, 1972), have similar molecular weights and amino acid sequences (I. D. Walker, personal communication) and contain about 8 half-cystine residues per mole. The proteins denoted as the γ -group do not appear to be keratins. Some (or all) of these proteins have a lower molecular weight than the keratins, a very different amino acid composition, including a low half-cystine content (I. D. Walker, personal communication), and do not form keratin fibrils *in vitro* (Kemp, 1972).

The results from quantitative gel electrophoresis described in the present work do not support a two-phase synthesis of the major feather keratin proteins during development of the embryonic feather. Within the present degree of accuracy the major keratin proteins are present in similar relative amounts of all stages of development, demonstrating a coordinated synthesis of the major keratin proteins. Synthesis of the minor γ -group of proteins begins well before that of the β -proteins, however, and it is possible that they were present in the sulphur-deficient protein fraction described by Malt and Bell (1965).

The increased sulphur content of feathers after 14 days of development observed by Malt and Bell (1965) is presumably due to the accumulation of the fibrous, α - and β -groups of feather keratin proteins, as these contain about eight half-cystine residues per mole.

Electron Microscope Observations on Feather Keratin Synthesis

The electron microscope visualization of keratin fibrils at various stages of feather development

confirmed the results of Matulionis (1970) and correlated well with the results from polyacrylamide gel electrophoresis of the extracted proteins. Since the fibrils are polymers of the keratin subunits detected by polyacrylamide gel electrophoresis, the observed correlation indicates that there cannot be a large pool of keratin monomers in the feather cells before fibrils can be seen. Consequently, electron microscopy and autoradiography provide a method of investigating the temporal relationship of DNA, RNA, and keratin synthesis within individual cells.

Keratin fibrils were not observed in cells which had incorporated [³H]thymidine immediately before examination, but were detected in cells which had incorporated [³H]uridine. Thus it would appear likely that the cells retain the ability to synthesize RNA after the onset of keratin synthesis, but that DNA synthesis and keratin synthesis are mutually exclusive events within the one cell. The latter conclusion is still subject to the limitation of the sensitivity of detection of keratin fibrils and relies on the absence of a large pool of keratin monomers in the feather cells before fibrils can be seen. Nevertheless, a similar relationship exists between DNA synthesis (and hence mitosis) and overt cytodifferentiation in certain other systems, for example, the embryonic chick myoblast (Holtzer, 1970). The results of Kischer and Furlong (1967) support the conclusion that DNA synthesis ceases by the time the major onset of feather keratin synthesis begins.

It is evident from the polyacrylamide gel profiles of keratin proteins observed in the present work that feather tissue samples can be obtained at different developmental stages, from that at 11–12 days when keratin synthesis is negligible, to that at later stages (14–15 days) when the predominant activity is synthesis of keratin. The quantitative methods described for the study of keratin synthesis during this transition should therefore be particularly valuable for the analysis of the mechanisms controlling the onset of keratin synthesis.

Furthermore, the ability of cultured feathers to incorporate [¹⁴C]leucine into a spectrum of proteins at least resembling the *in vivo* situation at each age of development should facilitate studies on the control of keratin synthesis. Such a system for the study of keratin synthesis has not previously been available.¹

¹Since this paper was submitted, the study of another keratin-synthesizing tissue has been reported by K. B.

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