2. There is a lag in growth and oxygen uptake on transferring cells from growth on glucose to an amino acid medium during which the enzymes for amino acid catabolism are formed.

3. In the presence of glucose, or any of a variety of alternative energy sources, adaptation for amino acid breakdown is repressed.

4. The effect of glucose on the induction of individual enzymes in the catabolic pathways of tyrosine and histidine has been measured with cell-free extracts.

5. *p*-Hydroxyphenylpyruvate hydroxylase and homogentisate oxygenase, the second and third enzymes of tyrosine degradation, are repressed by glucose, but tyrosine transaminase, the first enzyme, is neither induced by tyrosine nor repressed by glucose.

6. Histidase and urocanase, the first enzymes in the histidine pathway, are repressed by glucose.

7. Utilization of tyrosine or histidine by fully adapted cells is not decreased in the presence of glucose or other metabolic repressors except acetate.

8. Induction and repression of amino acid oxidation are complementary control mechanisms by which the large enzymic potential of this organism is regulated.

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# Soluble Derivatives of Feather Keratin

## 1. ISOLATION, FRACTIONATION AND AMINO ACID COMPOSITION

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Recent studies of the fine structure of feather keratin (Filshie & Rogers, 1962; Rogers & Filshie, 1962, 1963) have shown the existence of a microfibril–matrix structure similar to that observed in α-keratin. In α-keratin this structure has been related to proteins of lower and higher sulphur content (Birbeck & Mercer, 1957*a*, *b*, *c*; Rogers, 1959). The study of the soluble proteins should provide evidence as to whether two similar groups of proteins also exist in feather keratin.

Previous studies on soluble proteins from feathers (Ward, High & Lundgren, 1946; Rougvie,

1954; Woodin, 1954, 1956) have been carried out on whole feathers. Schroeder, Kay, Lewis & Munger (1955) have shown that the amino acid compositions of the morphological parts of turkey feathers are different and emphasized that in studies of feather keratin definite portions rather than whole feathers should be investigated in order to obtain meaningful results. This would be expected to be important in any critical examination of the homogeneity of the soluble feather proteins. Considerable progress has been made in the last decade in the isolation of soluble protein derivatives from wool keratin (see Gillespie, O'Donnell, Thompson & Woods, 1960; Woods, 1961), and the application of such methods to the morphological parts of the feather is reported in the present paper.

## EXPERIMENTAL

### *Preparation of feather parts*

Wing feathers from adult white Leghorn hens were used for this work. The feathers were washed in a hot dilute detergent solution at neutral pH, and rinsed in warm tap water and then distilled water. Any pulpy protein in the calamus was squeezed out and the feathers were air-dried. They were then extracted with several changes of light petroleum until a small portion of the solvent gave no residue when allowed to evaporate on a watch-glass. This was followed by two rinses with 95% (v/v) ethanol, one with distilled water and two more with 95% ethanol, after which the feathers were allowed to air-dry. The separation into the four main parts was carried out as described by Schroeder *et al.* (1955). These authors describe the anatomy of the feather and we have followed the system of nomenclature given in their paper. The proportions of the various components (excluding the distal portion of the rachis which could not be separated from medulla) expressed as percentages of the whole feather were: calamus, 19; barbs, 32; medulla, 13; rachis, 36.

### *Extraction of soluble proteins*

*Reduction and alkylation.* In order to determine the optimum conditions for extraction the effects of temperature, time, pH and concentration of reducing agent were studied for rachis. The reducing agent used was sodium mercaptoacetate with a liquor:feather ratio of 100:1 (v/w), and the protein concentrations were obtained by determination of nitrogen by the Kjeldahl method. The mercaptoacetic acid (Hopkin and Williams Ltd.) was redistilled under reduced pressure (12 mm. Hg) to eliminate thiol esters (see White, 1960). Below pH 9·5 less than 1% of the protein was extracted at 25° and there was a rapid increase in the amount of protein extracted between pH 9·5 and 10·5. The amount extracted decreased at mercaptoacetate concentrations above 0·2M. For studies on the effect of time and temperature on the extraction process, the conditions used were 0·1M-mercaptoacetate and pH 11·0, at temperatures of 2°, 25° and 40°. The effect of temperature on the rate of extraction was similar to that found for the extraction of wool proteins (Gillespie & Lennox, 1955). The activation energy calculated from the initial rates of solution as a function of temperature was about 13 kcal./mole. This compares with the value of 13·7 kcal./mole for the extraction of wool proteins recalculated from the results given by Gillespie & Lennox (1955) (the value of 7·4 kcal./mole reported by them contains an arithmetical error). Values of this order for the activation energy have been found for many processes occurring in keratin fibres and have been ascribed to a diffusion process subject to considerable restriction within the fibre (Alexander & Hudson, 1954).

From the foregoing experiments the mildest conditions consistent with 85–90% extraction of protein were chosen as follows: liquor:feather ratio, 100:1 (v/w); 0·1M-sodium mercaptoacetate, pH 11·0; 2° for 30–36 hr. The sodium mercaptoacetate solution was saturated with $N_2$ at 2° and an atmosphere of $N_2$ was maintained in the flask during the extraction. After extraction the residue was filtered and washed with a small quantity of 0·01M-sodium mercaptoacetate, pH 11·0. To stabilize the –SH groups against reoxidation the combined extract and washings were treated with sodium iodoacetate at 20°. Iodoacetic acid, in amount equivalent to 3 times the molar concentration of the mercaptoacetate, was adjusted to pH 6·0, added to the extract and the pH maintained at 9·5 until the nitroprusside test was negative. The reaction was always complete in less than 15 min. Sodium sulphite was then added to destroy the excess of iodoacetate, and the reaction products were removed by dialysis against distilled water at 2° in 18/32 Visking cellophan tubing (the protein diffuses through tubing of greater pore size than this). Alternatively, the excess of iodoacetate was removed by precipitating the protein with 7½% (w/v) trichloroacetic acid. To ensure complete reduction and to eliminate the possibility of mixed disulphide formation, a further treatment for 30 min. with 0·1M-sodium mercaptoacetate, pH 11·0, at 20° was given followed by alkylation with sodium iodoacetate. If the insoluble residue from the extraction was required it was also treated with sodium iodoacetate, washed by soaking with several changes of distilled water and air-dried. By this procedure about 85–90% of the rachis, barbs and medulla could be readily obtained in solution. Further extraction of the rachis residues indicated that the maximum solubility was about 92%. With calamus the foregoing procedure extracted only about 40% of protein and longer times or higher temperatures were required to achieve complete extraction. It was found preferable to shorten the extraction times for calamus by incorporating urea (8M) into the extraction medium. In the presence of 8M-urea, 0·1M-sodium mercaptoacetate at pH 9·3 dissolved 80–85% of the calamus in 24 hr. at 20°. The protein was alkylated as described above. Proteins prepared by the reduction and alkylation procedure are referred to as *S*-carboxymethyl derivatives.

*Extraction with cuprammonium sulphite.* The procedure followed was similar to that of Swan (1961), except that we have found that 8M-urea is not necessary for solution of the protein. The percentage of protein dissolved was determined from the dry weights of the washed residues. Studies on the effect of temperature on the rate of solution gave results almost identical with the first method, i.e. an activation energy of about 13 kcal./mole. The procedure adopted to prepare soluble proteins was as follows: two solutions, containing (i) $CuSO_4$ (0·1M) plus $NH_3$ (0·5N),

and (ii) $Na_2SO_3$ (0·2M) plus $NH_3$ (0·3N), were mixed in equal proportions and the feather was extracted with this mixture for 40 hr. at 2°. For a liquor:feather ratio of 100:1 (v/w), 85–90% of the protein dissolved. The insoluble residue was filtered off and the protein solutions were freed from copper by passing them through a column of cation-exchange resin [Dowex AG50W (X8; $NH_4^+$ form)]; the eluate was made 0·05% with respect to EDTA (disodium salt) and then dialysed against successive changes of distilled water. The protein was concentrated by freeze-drying and dissolving in dilute borate. Analysis for copper, determined colorimetrically with sodium diethyldithiocarbamate (Allport & Keyser, 1957), showed that copper was completely removed from the protein.

*Extraction at neutral* pH *with sulphite and tetrathionate.* The procedure was based on the method of Bailey & Cole (1959) for the oxidative sulphitolysis of proteins at neutral pH. The extractant consisted of urea (8M), $Na_2SO_3$ (0·2M), $Na_2S_4O_6$ (0·1M) and tris (0·1M) adjusted to pH 7·5 with HCl. The liquor:feather ratio was 100:1 (v/w) and after 24 hr. at 28° further additions of $Na_2SO_3$ and $Na_2S_4O_6$ were made to bring their total concentrations to 0·4M and 0·2M respectively, and the pH was readjusted to 7·5. After a further 48 hr. at 28°, 80% had dissolved. The insoluble material was filtered off, the reactants were removed by dialysis, and the protein was concentrated if necessary by freeze-drying.

Derivatives prepared by sulphitolysis are termed '*S*-sulpho-proteins' (Swan, 1961).

*Preparation of soluble protein by oxidation.* Performic acid reagent was prepared by mixing 1 vol. of 30% (v/v) $H_2O_2$ and 9 vol. of 98–100% (v/v) formic acid and allowing to stand 1–2 hr. at room temperature (Toennies & Homiller, 1942). Feather rachis (1 g.) was oxidized with this reagent (30 ml.) at 2° for 22 hr. The excess of performic acid was removed by dilution and dialysis against cold distilled water, as described by O'Donnell & Thompson (1962) for wool. The contents of the dialysis bags were adjusted to pH 8·5 with NaOH on the pH-stat and stirred for 2 hr. The insoluble residue was removed by centrifugation and the protein concentrated by freeze-drying. The protein extracted was 88% of the starting material. These protein derivatives are referred to as 'keratoses', in common with the protein derivatives produced by the oxidation of wool keratin.

## *Analytical methods*

*Nitrogen.* This was determined by the semimicro-Kjeldahl method. Nitrogen contents for the soluble proteins were determined by first dialysing against 0·2M-NaCl. Samples of the diffusate and contents of the dialysis bag were taken for determinations of nitrogen and of dry weight (dried to constant weight in a vacuum oven at 105°), and correction was made for the volume of salt solution excluded by the protein when calculating the dry weight.

*Sulphur.* Three methods were used: determination as $BaSO_4$ by the Carius method with 0·2 g. samples, and microdeterminations by both the methods of Schöniger (1956) and Zimmermann (1947). Good agreement was obtained between all three methods, and where more than one method was used only the mean values are reported.

*Ash.* The samples were incinerated in a stream of oxygen at 800°.

*Thiol and disulphide.* These were determined polarographically (see Leach, 1960*a*, *b*).

*Amino acid analysis.* Protein samples (50 mg.) were hydrolysed for 24 hr. with 10 ml. of 6N-HCl under reflux. The hydrolysate was evaporated *in vacuo* at below 0° and redissolved in distilled water. After filtering to remove humin the nitrogen content of this solution was estimated and its amino acid composition determined with the Spinco automatic amino acid analyser (model 120) as described by Spackman, Stein & Moore (1958). Tryptophan was determined separately by the method of Spies & Chambers (1949).

*Amide determinations.* The procedure of Leach & Parkhill (1956) involving hydrolysis with 12N-HCl at 37° was used.

*Acyl groups.* *O*-Acyl groups were determined by the method involving alkaline hydrolysis at pH 10 (Herriott, 1935), and total acyl groups by the acid hydrolysis procedure of O'Donnell, Thompson & Inglis (1962).

*Ultraviolet-absorption curves.* These were measured on a Beckman model DK2 spectrophotometer.

*Electrophoresis.* The proteins were examined by moving-boundary electrophoresis in an apparatus made by LKB Produktor, Stockholm. Buffers of *I* 0·1 were used: tris–HCl–NaCl at pH 7·4; diethylbarbituric acid (veronal)–NaOH at pH 8·6; β-alanine–NaOH at pH 11·0.

*Solubility curves.* The solubility of the extracted proteins as a function of pH was determined by dialysing 5 ml. samples of protein solution (0·5%) against 50 ml. portions of phosphate–acetate–NaCl buffer solutions (*I* 0·2) at various pH values for 24 hr. at 2° with continual rocking. For pH values below 1·5 mixtures of NaCl and HCl were used. After equilibration the contents of the dialysis bags were centrifuged at 2°, the supernatant was diluted 1:4 with 50% (v/v) acetic acid (to eliminate any residual turbidity) and the extinction was measured at 277 mμ.

To determine the solubility of the proteins as a function of ethanol and $(NH_4)_2SO_4$ concentrations, appropriate volumes of protein solution and precipitant were mixed in glass-stoppered tubes to give final volumes of 10 ml., after which the tubes were gently rocked at 2° for 24 hr. These precipitations were carried out in a final concentration of 0·01M-sodium acetate buffer, pH 6·0, and the ethanol precipitations were carried out in the presence of 0·02M-zinc acetate. The insoluble protein was centrifuged at 2° and the extinctions of the supernatants were measured at 277 mμ.

*Chromatography.* This was carried out at 20° on columns (0·9 cm. × 15·0 cm.) of DEAE-cellulose equilibrated with 0·01M-tris buffer, pH 7·4. Both stepwise and gradient elution with increasing KCl concentration were used. For gradient elution a mixer with a 120 ml. mixing chamber of the type described by Moore & Stein (1954) was used. Fractions (approx. 4 ml.) were collected by means of a drop counter, and the extinctions measured at 277 mμ.

## RESULTS

*Analyses of feather parts.* Tables 1 and 2 give nitrogen, sulphur and amino acid analyses of the feather parts. The results in Table 2 refer to one hydrolysis time (24 hr.) and thus are uncorrected for possible destruction of such amino acids as serine, threonine and tyrosine. The thiol-plus-disulphide content (determined polarographically)

together with the methionine accounts for all of the sulphur of rachis and calamus within experimental error, but in the barbs and medulla there appears to be some sulphur unaccounted for. It is possible that some inorganic sulphur is present, as suggested by Schroeder *et al.* (1955) (see also Lindley, 1948.)

*Electrophoresis of soluble feather parts.* *S*-Carboxymethyl-rachis was submitted to electrophoresis in buffers (*I* 0·1) at pH values 7·4, 8·6 and 11·0. Heterogeneity was evident at all pH values; however, the boundaries in the tris buffer were incompletely resolved and resolution improved progressively as the pH was increased. For a comparison of the soluble proteins of the feather parts electrophoresis was carried out in β-alanine–sodium hydroxide buffer, pH 11·0 (Fig. 1). Only the ascending patterns are shown since these were much sharper and showed better resolution of

Table 1. *Nitrogen, sulphur and ash contents of feather parts*

Experimental details are given in the text. Nitrogen and sulphur contents were calculated on a moisture-free but not an ash-free basis, and are given as means ± S.E.M., with the numbers of determinations in parentheses. The ash contents were all decreased to approx. 0·2% on washing with dilute acid. Only single determinations for ash contents were made.

| Feather part | Nitrogen content (%) | Sulphur content (%) | Ash content (%) |
|---|---|---|---|
| Rachis | 16·62 ± 0·055 (15) | 2·47 ± 0·029 (9) | 0·87 |
| Calamus | 16·40 ± 0·052 (9) | 2·32 ± 0·011 (8) | 0·80 |
| Barbs | 16·28 ± 0·075 (6) | 2·85 ± 0·035 (7) | 0·86 |
| Medulla | 16·36 ± 0·075 (13) | 2·40 ± 0·019 (8) | 0·77 |

Table 2. *Amino acid compositions of feather morphological parts*

The hydrolysis of the feather parts was carried out by refluxing with 6N-HCl for 24 hr. Other experimental details are given in the text. The results are uncorrected for destruction.

| | Amino acid composition (μmoles/g.) | | | |
|---|---|---|---|---|
| | Rachis | Calamus | Barbs | Medulla |
| Ala | 840 | 729 | 446 | 655 |
| Amide* | 963 | 857 | 858 | 965 |
| Arg | 370 | 371 | 384 | 389 |
| Asp | 547 | 534 | 519 | 537 |
| CySH* | 39 | 23 | 36 | 24 |
| CyS* | 722 | 686 | 732 | 670 |
| Glu | 671 | 698 | 686 | 684 |
| Gly | 1326 | 1171 | 970 | 1156 |
| His | 23 | 20 | 17 | 24 |
| Ileu | 308 | 280 | 383 | 313 |
| Leu | 802 | 664 | 605 | 720 |
| Lys | 62 | 52 | 71 | 65 |
| Met | 8 | 13 | 15 | 18 |
| Phe | 301 | 319 | 296 | 330 |
| Pro | 948 | 885 | 1056 | 891 |
| Ser | 1365 | 1299 | 1236 | 1363 |
| Thre | 401 | 345 | 437 | 366 |
| Try* | 72 | 77 | 22 | 75 |
| Tyr | 139 | 143 | 118 | 147 |
| Val | 756 | 673 | 705 | 684 |
| % of N accounted for | 101 | 95 | 93 | 95 |
| % of S accounted for | 95 | 97 | 84 | 92 |

* Determined separately, as described in the text.

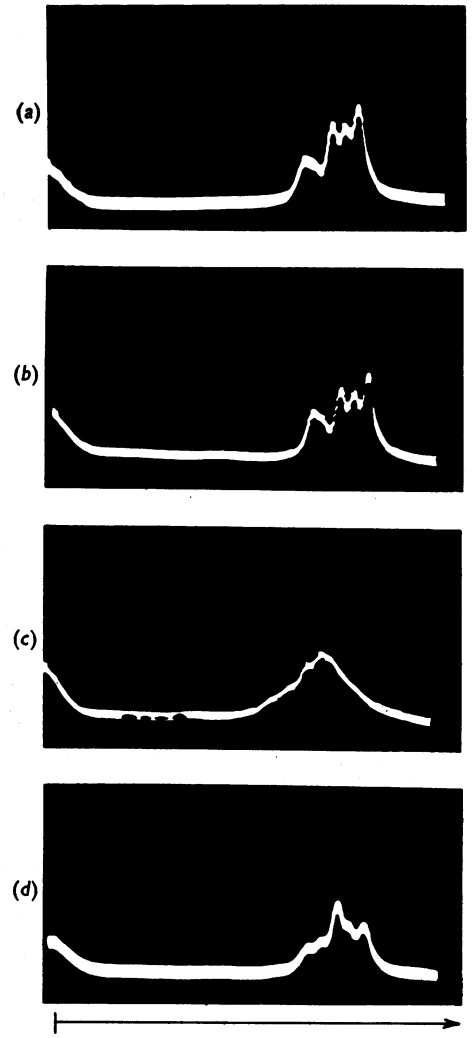


Fig. 1. Electrophoresis of the soluble *S*-carboxymethyl derivatives of feather parts. The electrophoresis was carried out in β-alanine–NaOH buffer, pH 11·0 and *I* 0·1, with a potential gradient of 5·9 v/cm. The concentrations and times of electrophoresis were: (*a*) Rachis: 1·07%; 270 min. (*b*) Calamus: 1·19%; 270 min. (*c*) Barbs: 1·09%; 244 min. (*d*) Medulla: 1·0%; 270 min.

components than the descending boundaries. The mobilities of the ascending boundaries are given in Table 3.

*Comparison of various extraction procedures.* A comparison was made of the soluble derivatives obtained with different methods of breaking the cystine cross-links in the feather rachis. Fig. 2 gives the electrophoretic patterns of *S*-carboxymethyl-rachis, *S*-sulpho-rachis prepared by the two methods of sulphitolysis described above, and rachis keratose. Because of the instability of the $-S \cdot SO_3^-$ group at alkaline pH values (see Thompson, 1960), electrophoresis was done at pH 8·6 in veronal buffer for these derivatives and the patterns were compared with those for *S*-carboxymethyl-rachis in the same buffer. The *S*-carboxymethyl and *S*-sulpho derivatives show the same components in similar proportions. Comparison with the patterns of the rachis keratose shows that there is much poorer definition of components for the keratoses than for either of the other two derivatives.

To test for the completeness and specificity of the methods used to rupture the –S·S– bonds determinations were made of the total sulphur, residual –S·S– and –SH groups, and, where applicable, *S*-carboxymethylcysteine and cysteic acid. In all cases the disulphide-plus-thiol content was zero. The sulphur of the *S*-carboxymethyl-rachis (four determinations gave a mean of 2·44 %) could all be accounted for as *S*-carboxymethyl-cysteine (Table 4) and methionine, whereas for the rachis keratose 90 % of the sulphur could be accounted for as cysteic acid and methionine. The cysteic acid values were not corrected for any destruction during hydrolysis. For the *S*-sulpho-rachis the mean of four determinations gave a sulphur content of 4·40 %, whereas the expected value when every –S·S– group is converted into two $-S \cdot SO_3^-$ groups is 4·65 %, indicating complete conversion within the experimental accuracy of the sulphur determinations.

Table 3. *Mobilities of electrophoretic components of soluble* S*-carboxymethyl derivatives of feather parts*

Moving-boundary electrophoresis was carried out in β-alanine–NaOH buffer, pH 11·0 and *I* 0·1. The protein concentrations were as given in Fig. 1. All components are negatively charged.

| Morphological part | $10^5 \times$ Mobility (ascending) ($cm.^2 sec.^{-1} v^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| Rachis | 4·79 | | 4·99 | | 5·40 | 5·67 | 5·95* |
| Calamus | | 4·90 | 5·06 | | 5·41 | 5·65 | 5·93* |
| Medulla | 4·69 | 4·93 | | 5·26* | 5·46 | 5·72 | |
| Barbs | | | | | | 5·72* | |

* Main component; with barbs the other components were not sufficiently resolved to measure their mobilities.

*Chromatography on diethylaminoethylcellulose.* Stepwise elution at pH 7·4 was carried out over the range 0–1·0 M-potassium chloride. The increments of potassium chloride concentration were 0·025 M over the range 0·15–0·3 M. Three hold-up volumes were used for each increment. As shown in Fig. 3 (*a*)

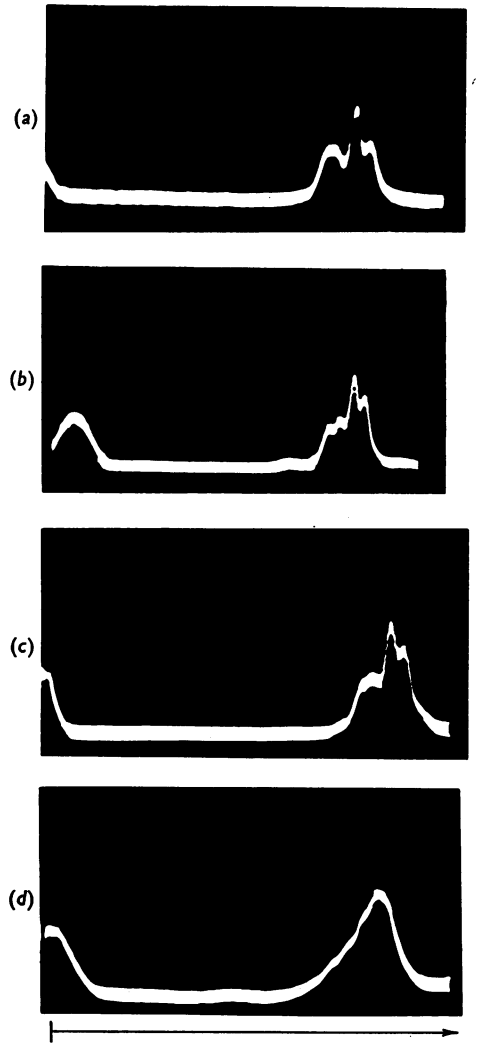


Fig. 2. Ascending electrophoretic patterns of soluble derivatives of feather rachis prepared by different procedures. The electrophoresis was carried out: (*a*), (*b*) and (*c*) in veronal–NaOH buffer, pH 8·6 and *I* 0·1, with a potential gradient of 6·62 v/cm.; and (*d*) in β-alanine–NaOH buffer, pH 11·0, with a potential gradient of 5·83 v/cm. The concentrations and times of electrophoresis were: (*a*) *S*-Carboxymethyl-rachis: 1·13 %; 300 min. (*b*) *S*-Sulpho-rachis prepared by the cuprammonium sulphite method: 1·34 %; 270 min. (*c*) *S*-Sulpho-rachis prepared by the sulphite–tetrathionate method: 1·22 %; 270 min. (*d*) Rachis keratose: 1·26 %; 257 min.

four peaks were eluted between 0·175M- and 0·3M-potassium chloride, by which stage 96% of the protein had been eluted from the column. However, the extinction did not return to the base line with each increment. Gradient elution was also carried out over the range 0–0·5M-potassium chloride, and these results are shown in Fig. 3(*b*). Again, under these conditions, trailing of the peaks was evident. These results are only of a preliminary nature and are intended to demonstrate that the electrophoretic heterogeneity of these preparations is also evident in their behaviour on DEAE-cellulose chromatography.

*Solubility curves.* The solubilities of *S*-carboxymethyl-rachis and rachis keratose as a function of pH are shown in Fig. 4, and for the *S*-carboxymethyl-rachis as functions of ammonium sulphate concentration and of ethanol concentration (in the presence of 0·02M-zinc acetate) in Fig. 5. The shift of the rachis keratose solubility curve to lower pH values with respect to the *S*-carboxymethyl-rachis solubility curve (Fig. 4) is due to the presence of the strongly acidic $-SO_3^-$ groups in the keratose. With ammonium sulphate, precipitation occurs over a narrow range of salt concentration, but the breadth of the precipitation region in the pH and ethanol solubility curves is indicative of heterogeneity, and we have attempted fractionation by making use of these findings.

*Ethanol fractionation.* Three fractions were prepared from *S*-carboxymethyl-rachis by fractional precipitation at 2°, first with 5% (v/v) and then with 12·5% (v/v) ethanol at pH 6·0 [sodium acetate (0·01M) plus zinc acetate (0·2M)]. The electrophoretic patterns of these fractions, representing 7% ($P_1$), 43% ($P_2$) and 50% ($S_2$) respectively of the soluble rachis, are shown in Fig. 6, and Table 4 gives the amino acid compositions of the original *S*-carboxymethyl-rachis and of the three ethanol fractions derived from it. With the exception of serine, *S*-carboxymethylcysteine and amide the values in Table 4 for the unfractionated *S*-carboxymethyl-rachis can all be accounted for by summing the values for the fractions. The higher values for serine and *S*-carboxymethylcysteine for the unfractionated material indicate that less

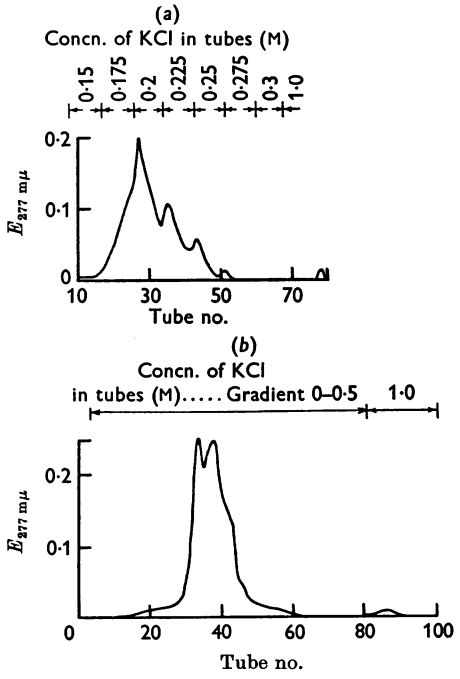


Fig. 3. Chromatography of *S*-carboxymethyl-rachis on DEAE-cellulose at 20° in 0·01M-tris–HCl buffer, pH 7·4. The column size was 0·9 cm. × 15 cm., and the fraction size was 4 ml. (*a*) Stepwise elution. (*b*) Gradient elution.

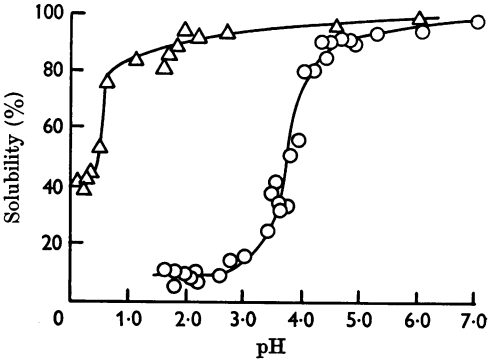


Fig. 4. Solubility of *S*-carboxymethyl-rachis (○) and rachis keratose (△) as a function of pH.

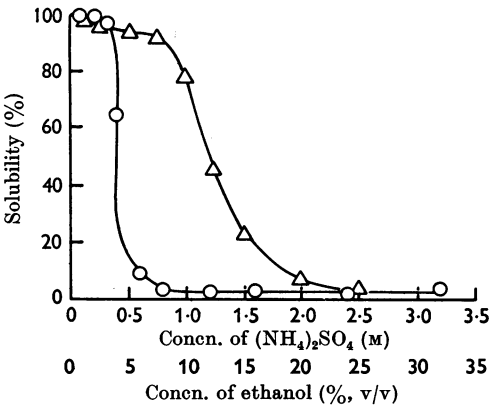


Fig. 5. Solubility of *S*-carboxymethyl-rachis in $(NH_4)_2SO_4$ at pH 6 (○) and in ethanol in the presence of 0·02M-zinc acetate (△).

destruction of these amino acids occurred on hydrolysis; this is consistent with the lower amide value.

*Acid fractionation.* *S*-Carboxymethyl-rachis was divided into two fractions by precipitating 60 % of the soluble proteins at pH 3·7 and 2° in the phosphate–acetate–sodium chloride buffer. The electrophoretic patterns of the two fractions are given in Fig. 7. Rachis keratose was also divided into fractions by precipitation with 2N-hydrochloric acid in the presence of 0·2M-sodium chloride, the precipitate fraction representing 55 % of the soluble proteins. Sulphur and nitrogen analyses were done on all the precipitate and supernatant fractions and these are summarized in Table 5. We have also included for comparison the S:N ratios for fractions produced by acid precipitation of the soluble proteins from wool keratin.

*Fractional extraction.* With wool keratin it is possible to extract preferentially proteins of high sulphur content and to extract the low-sulphur proteins subsequently (Gillespie & Lennox, 1955; Gillespie, 1962). Fractional-extraction experiments were therefore attempted on feather rachis. Rachis was extracted at 25° for 3½ hr. with 0·1M-sodium mercaptoacetate at pH 11, and 40 % of the protein dissolved; the supernatant was removed and the rachis extracted with a fresh sodium mercaptoacetate solution for 18 hr. at 2°, and a further 48 % of the rachis dissolved. The residue was again extracted with fresh sodium mercaptoacetate at 2° for 48 hr. and macerated in a Waring Blendor to give a further 3 % of dissolved protein. The fractions were converted into the *S*-carboxymethyl derivatives in the usual way. Electrophoresis of the fractions showed little difference between the two major portions; the second extract was slightly enriched in the slowest-moving peak. However, the last 3 % extracted was different from the other fractions in that its electrophoretic diagram showed a preponderance of slower-moving material and was similar to that of the $P_1$ fraction from the ethanol fractionation. The smallest fraction was also precipitated on dialysis against distilled water, being different in this regard to the other fractions. Its amino acid composition was similar to that of fraction $P_1$ and closer to that of the undissolved residue, which has higher lysine, histidine, tyrosine and methionine contents, and lower serine and proline contents, than the major part of the soluble proteins.

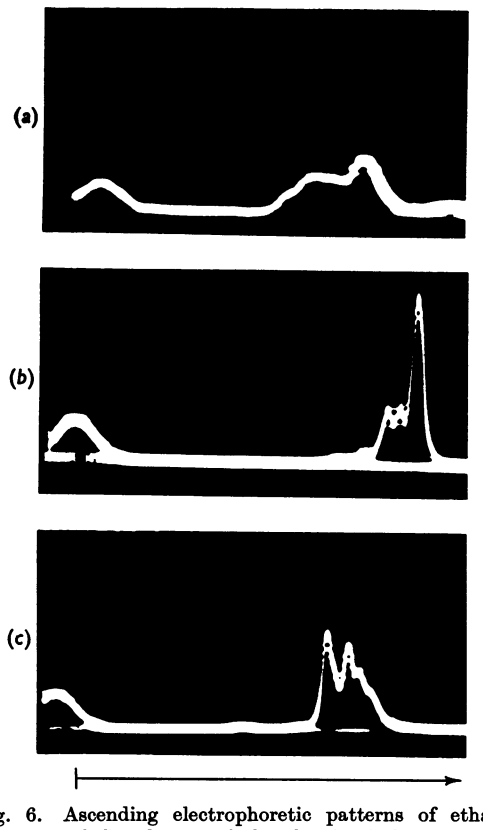


Fig. 6. Ascending electrophoretic patterns of ethanol fractions of *S*-carboxymethyl-rachis in β-alanine–NaOH buffer, pH 11·1 and *I* 0·1, with a potential gradient of 5·90 v/cm. The concentrations and times of electrophoresis were: (*a*) Fraction $P_1$: 0·44 %; 210 min. (*b*) Fraction $P_2$: 1·27 %; 270 min. (*c*) Fraction $S_2$: 1·33 %; 270 min.

*Nitrogen content and ultraviolet-absorption spectra.* The nitrogen content of unfractionated *S*-carboxymethyl-rachis determined on a dry-weight basis after dialysis was $16{\cdot}1_8$ % (mean of four determinations). The ultraviolet-absorption curves of *S*-carboxymethyl-rachis and rachis keratose are given in Fig. 8. The extinction for the *S*-carboxymethyl-rachis shows a maximum at 277 mμ in the region expected for unfolded proteins, but $E^{1\%}_{1\,cm.}$ varied between 6·4 and 7·5 in four different preparations. This is probably due to variations in the amount of protein extracted; as we have shown above the final few per cent of protein dissolved when one reaches over 85 % solution has a much higher tyrosine content than the bulk of the extracted protein. The curve for the rachis keratose is not typical of proteins in that no maximum is observed in the region of 280 mμ. Performic acid is known to convert tryptophan into a variety of oxidation products, and also to modify methionine residues, and it seems that these modifications are responsible for the protein ultraviolet-absorption curve in this region. The ultraviolet-absorption curve of *S*-sulpho-rachis prepared by the cuprammonium sulphite method was identical with that for *S*-carboxymethyl-rachis. On the other hand the curve for the *S*-sulpho-rachis prepared by the tetrathionate–

Table 4. *Amino acid compositions of* S-*carboxymethyl-rachis fractions*

The hydrolysis of the *S*-carboxymethyl-rachis fractions was carried out by refluxing with 6N-HCl for 24 hr. Other experimental details are given in the text. The results are uncorrected for destruction and are calculated on the basis of a nitrogen content of 16·2% for all the fractions.

| | Amino acid composition (μmoles/g.) | | | | |
|---|---|---|---|---|---|
| | Soluble *S*-carboxymethyl-rachis | | | | |
| | Un-fractionated | $P_1$ fraction | $P_2$ fraction | $S_2$ fraction | Insoluble residue |
| Ala | 793 | 596 | 690 | 916 | 584 |
| Amide | 957*; 989† | 1014* | 1104* | 967* | 881* |
| Arg | 334 | 405 | 370 | 313 | 444 |
| Asp | 504 | 559 | 553 | 441 | 677 |
| CyS | 0 | 86 | 0 | 0 | 264* |
| Glu | 630 | 717 | 701 | 493 | 842 |
| Gly | 1285 | 1066 | 1163 | 1436 | 811 |
| His | Trace | 50 | 0 | 0 | 120 |
| Ileu | 283 | 270 | 285 | 281 | 366 |
| Leu | 724 | 589 | 603 | 887 | 710 |
| Lys | 13 | 115 | < 6 | 10 | 450 |
| Met | Trace | 32 | 0 | 0 | 114 |
| Phe | 302 | 293 | 349 | 273 | 227 |
| Pro | 970 | 765 | 926 | 1048 | 428 |
| $CyS{\cdot}CH_2{\cdot}CO_2H$ | 762 | 530 | 710 | 688 | 0 |
| Ser | 1392 | 1051 | 1154 | 1211 | 542 |
| Thre | 365 | 366 | 336 | 395 | 369 |
| Try | 66‡ | § | § | § | § |
| Tyr | 107 | 271 | 124 | 94 | 232 |
| Val | 743 | 614 | 724 | 731 | 585 |
| % of N accounted for | 98 | § | § | § | § |
| % of S accounted for | 100 | § | § | § | § |

* Determined on hydrolysate applied to automatic amino acid analyser.
† Determined by hydrolysis with 12N-HCl for 120 hr. at 37° (see the text).
‡ Determined separately, as described in the text.
§ Not determined.

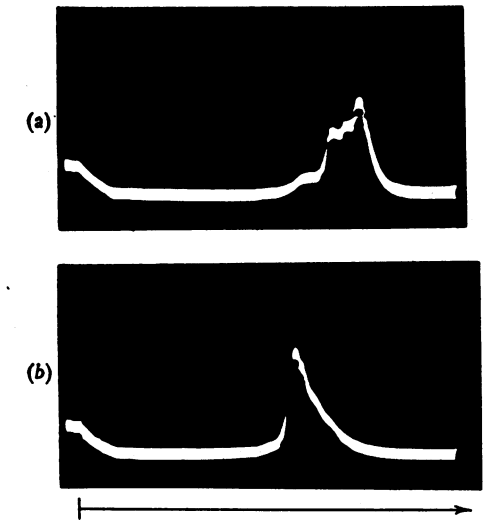


Fig. 7. Ascending electrophoretic patterns of acid fractions of *S*-carboxymethyl-rachis in β-alanine–NaOH buffer, pH 11·0 and *I* 0·1, with a potential gradient of 5·95v/cm. The concentrations and times of electrophoresis were: (*a*) Precipitate fraction: 0·74%; 240 min. (*b*) Supernatant fraction: 0·73%; 240 min.

Table 5. *Sulphur : nitrogen ratio of soluble feather-rachis fractions*

Experimental details are given in the text.

| | Ratio of S content (%) to N content (%) | |
|---|---|---|
| | Precipitate | Supernatant |
| *S*-Carboxymethyl-rachis | | |
| Ethanol fractionation | $0{\cdot}13_3$ | $0{\cdot}13_7$ |
| Acid fractionation | $0{\cdot}12_9$ | $0{\cdot}13_1$ |
| Rachis keratose | | |
| Acid fractionation | $0{\cdot}13_1$ | $0{\cdot}13_8$ |
| *S*-Carboxymethyl-wool and wool keratose | | |
| Acid fractionation | $0{\cdot}14_3$ | $0{\cdot}40_0$ |

sulphite method was similar to that for rachis keratose, and analysis showed that the tryptophan content was decreased by 75%. Similar changes in the ultraviolet-absorption characteristics have also been observed when the –S·S– bonds of wool and bovine serum albumin have been split with tetrathionate and sulphite according to the method used in the present work.

*Acyl groups.* Both the native rachis and the unfractionated *S*-carboxymethyl-rachis contain

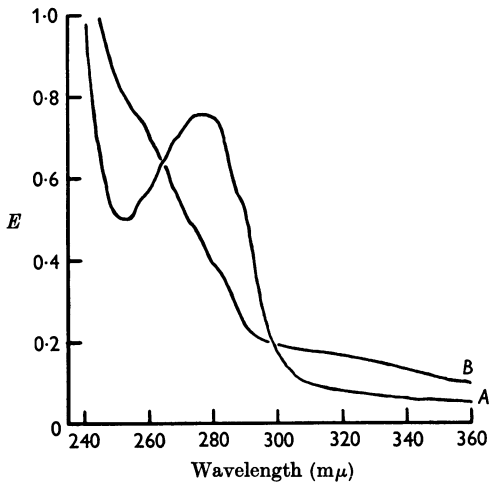


Fig. 8. Ultraviolet-absorption spectra of (*A*) *S*-carboxymethyl-rachis (concn. 0·094%) and (*B*) rachis keratose (concn. 0·072%).

identical amounts of total acyl groups to the extent of 1·30 moles/$10^4$ g. Since the *O*-acyl contents were shown to be zero, the acyl groups must be attached to the primary amino groups.

## DISCUSSION

The only previous amino acid analyses on the morphological components of feathers appear to be those of Ward, Binkley & Snell (1955) (fowl feathers), Schroeder *et al.* (1955) (turkey feathers) and Krimm (1960) (fowl calamus). Ward *et al.* (1955) gave results obtained by microbiological assay techniques on barbs, calamus and shaft. The most complete analyses are those of Schroeder *et al.* (1955). Comparison of their findings with those in Table 2 shows that the differences between the parts are confined to the same amino acids for each species: alanine, cystine, glycine, isoleucine, proline and tyrosine. There does not appear to be as close a similarity between the rachis and calamus of fowl feathers as in those of turkey; however, for both species the barbs show the greatest differences from the other parts. This is also evident in the tryptophan content of the barbs, which is only about 25% of that in the other three parts. As might be expected small species differences exist between turkey and fowl feathers (cf. Schroeder *et al.* 1955), mainly in their glutamic acid, leucine, phenylalanine and tyrosine contents.

The electrophoretic patterns for the soluble *S*-carboxymethyl-proteins of feathers, representing more than 80% of the morphological parts in each case (Fig. 1), show the presence of five main peaks together with minor faster- and slower-moving components. Each part contains components of similar mobility (Table 3) but in different proportions. There is a close similarity between the patterns for the rachis and calamus and, as with the amino acid composition, the barbs show the greatest differences from the other three. The barbs show the poorest resolution on electrophoresis, and the spread of the pattern indicates an even more complex mixture than for the other parts. The reported electrophoretic homogeneity of rachis keratose (Woodin, 1954) may have been due to the poor resolution of derivatives prepared by the oxidation method (cf. Fig. 2).

The amino acid composition of the unfractionated *S*-carboxymethyl-rachis (Table 4) shows some differences from the partial analysis given by Woodin (1956) for rachis keratose but agrees in that lysine and histidine are lacking in the soluble extract. As might be expected the insoluble residue has much higher contents of these two amino acids and of methionine, which is also absent from the soluble proteins. It has much lower serine, cysteic acid, proline and glycine contents, and a higher tyrosine content, the values being generally consistent with those of Ward *et al.* (1946).

The electrophoretic patterns of the fractions produced by precipitation with ethanol–zinc acetate or acid (Figs. 6 and 7) suggest that at least a partial fractionation has been achieved by both methods. The identification of components on the basis of mobility is difficult in this system because of the closeness of the mobilities and the complexity of the mixture. However, it seems that the ethanol precipitate $P_2$ and the acid precipitate are enriched in the proteins of highest mobilities at the expense of the slower-moving components which are concentrated in the ethanol and acid supernatants. The ethanol precipitate $P_1$ is very heterogeneous and contains both the faster- and slower-moving minor peaks. From the amino acid compositions of the three ethanol fractions (Table 4) it is clear that at least a partial separation of chemically distinct protein chains has been achieved. The differences in composition between the three fractions are considerably greater than those between different species of whole feather or between the morphological parts of any one species. The lysine, histidine and methionine are concentrated in the least soluble fraction, $P_1$, the other two fractions being almost completely devoid of these residues. This is of interest since a *S*-carboxymethyl-keratin fraction from wool has been shown to have very low contents of these amino acids (Gillespie, 1963). All the *S*-carboxymethyl-proteins contain an excess of negatively charged groups. This arises from the conversion of –S·S– groups into two $-S\cdot CH_2\cdot CO_2^-$ groups each and in their di-

sulphide forms all fractions are in fact basic proteins. This situation is different from that in wool keratin, in which both acidic and basic fractions are present (Gillespie & Simmonds, 1960).

Table 5 shows that no significant fractionation of the soluble proteins has been achieved on the basis of differing sulphur contents; this is in marked contrast with the situation for soluble wool proteins and other α-keratins. With α-keratin there is strong evidence that the high-sulphur and low-sulphur proteins originate from a microfibril–matrix structure. The principal cytoplasmic component is believed to consist of microfibrils of keratin embedded in a non-fibrous matrix which is rich in sulphur (Birbeck & Mercer, 1957*a*, *b*, *c*; Rogers, 1959). Feather keratins have also been shown by electron microscopy (Filshie & Rogers, 1962; Rogers & Filshie, 1962, 1963) to have a microfibrillar structure, but the microfibrils are about 30 Å in diameter compared with 80 Å for α-keratins. On the basis of the reactions towards the electron-microscope staining procedures, the cystine content of the feather-keratin matrix appears to be not greatly different from that of the microfibrils. The inability to separate the soluble proteins into fractions of markedly differing sulphur contents lends support to this view. Further, fractional-extraction procedures that enable the soluble proteins of wool to be separated into high-sulphur and low-sulphur fractions do not give such a separation with feather rachis. Earland, Blakey & Stell (1962) have used the nomenclature α- and γ-keratoses to describe the fractions produced by acidification of feather keratose solutions. This nomenclature is based on the isolation of high-sulphur and low-sulphur proteins from oxidized α-keratin (Alexander & Earland, 1950). The use of the prefixes α- and γ- for feather keratoses does not appear to be justified in view of the arbitrary nature of the fractionation and the results given in Table 5, and the fact that at present the fractions have not been identified with any histological component of the rachis.

Films cast from aqueous solutions of reduced or oxidized feather keratin (Rougvie, 1954; Fraser & MacRae, 1959; Earland *et al.* 1962) show many of the X-ray-diffraction properties of the original material. Films, cast from the fractions produced by both the ethanol and acid precipitation procedures described above, have been examined by X-ray diffraction and electron microscopy by Filshie, Fraser, MacRae & Rogers (1964). They have shown that the ethanol fractions $P_2$ and $S_2$, and the acid precipitate and supernatant fractions, form oriented films. When observed in the electron microscope by the negative-staining technique it is seen that the unfractionated protein and the fractions derived from it polymerize spontaneously into fibrils with a great tendency to lateral aggregation. The formation of fibrils seems to be a slow process and occurs only from solutions that have stood for several weeks and show signs of gelation.

Krimm & Schor (1956) have postulated that the solubilization of feather keratin involves hydrolysis of susceptible peptide bonds adjacent to proline residues. In the present work widely different preparative procedures have yielded soluble feather keratins with similar components. The sulphitolysis method (Bailey & Cole, 1959) was carried out at pH 7·5 and under these conditions hydrolysis of peptide bonds is unlikely. In all our extraction methods complete fission of the –S·S– bonds and quantitative conversion into the required derivative has been achieved. No side reactions, such as the reaction of iodoacetate with residues other than cysteine (Gundlach, Stein & Moore, 1959), could be detected in the amino acid-analysis charts. However, the ultraviolet-absorption spectra show that tryptophan must have been greatly modified during the oxidation with performic acid and the reaction with sulphite and tetrathionate. The values for amide nitrogen (Tables 2 and 4) for the native and unfractionated *S*-carboxymethyl-rachis are not significantly different, indicating that amide bonds have not been hydrolysed even under the most alkaline (pH 11) extraction conditions.

*N*-Acyl groups are reported in both the native and *S*-carboxymethyl-rachis to the extent of 1·35 moles for each subunit (mol.wt. 10400; Harrap & Woods, 1964). The low lysine content of the soluble protein (approx. 0·1 mole/$10^4$ g.) indicates that the acyl groups are attached to the α-amino nitrogen atoms of the *N*-terminal end groups and explains the non-stoicheiometric quantity of end groups found by Woodin (1956). The presence of *N*-terminal end groups, even though masked, in both native and soluble feather proteins eliminates the possibility of a cyclic structure for the polypeptide chains as proposed by Woodin (1956).

## SUMMARY

1. The morphological parts (rachis, calamus, barbs and medulla) of fowl feathers show differences in amino acid composition of the same order as those reported for turkey feathers by Schroeder *et al.* (1955).

2. Soluble proteins have been prepared from the feather parts by reduction and alkylation, oxidative sulphitolysis and oxidation with performic acid. These proteins are all electrophoretically heterogeneous, showing at least five components.

3. The electrophoretic patterns of the *S*-carboxymethyl-proteins from the morphological parts

are all different, the barbs showing the greatest differences.

4. *S*-Carboxymethyl-rachis has been fractionated into three fractions with different amino acid compositions and electrophoretic patterns. Two of these fractions, representing about 80 % of the rachis, are almost devoid of lysine, histidine and methionine. These amino acids are concentrated mainly in the insoluble residue (approx. 8 % of the total), which also contains less proline and whose cystine content is about half that of the whole rachis.

5. It has not been possible to separate the soluble feather proteins into fractions of widely differing sulphur contents, as has been done for wool keratin, and this finding is discussed in relation to the microfibril–matrix structure of feather.

6. *N*-Acyl groups are present in feather rachis to the extent necessary to account for the previously reported lack of *N*-terminal end groups.

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