KERATIN FIBRES

II.* SEPARATION AND ANALYSIS OF MEDULLARY CELLS

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Summary

A mild method is described for the separation of the medullae of keratin fibres. The method consists of staining with gold, which is deposited preferentially in the medulla. The fibre is then disrupted by agitation in formic acid and the heavy medullary cells separated by density-gradient centrifugation. By means of model experiments on whole fibres and medulla produced by dissection of porcupine quills, it is concluded that (except for some oxidation of cystine to cysteic acid) a negligible amount of modification of the medulla results from the separation procedure.

Amino acid analyses of medulla from the hair of the rabbit, kangaroo, and platypus and the quill of the African porcupine are surprisingly similar, hence it is reasonable to average the results. The composition of the medulla is less like that of keratin fibres than any other protein histological component so far analysed. It contains about 1 residue in 4–5 of glutamic acid, 1 in 9 of citrulline, 1 in 12 of leucine, 1 in 14 of glycine, and only 1 in 35 of cystine.

The sodium hydroxide method hitherto used for the separation of medullae from keratin fibres is found to dissolve a considerable fraction of the medullary protein, thus leaving behind a fraction of very different amino acid composition. This method is obviously degradative and hence cannot be recommended.

I. Introduction

The medulla is a histological component situated near the centre of the fibre in many keratin fibres such as human hair, but does not occur in fine wools (Ryder 1963). Because of its very different composition and structure from the keratin of cuticle and cortex it has been separated from the latter by the use of reagents such as peracetic acid followed by ammonia or aqueous caustic alkali, which dissolve the keratin and leave the medullary cells behind (Blackburn 1948; Matoltsy 1953; Ross 1955; Rogers 1962, 1964). However, it is shown in this paper that the use of such severe treatments produces considerable modification of the medulla. It is therefore desirable to develop a mild method for the separation of medulla from keratin fibres. This also presents the opportunity to extend the techniques which have been developed in this laboratory for the separation of the histological components of keratin fibres, viz. epicuticle (King and Bradbury 1968), cuticle, cortical cells, and cell membrane complex (Bradbury and Chapman 1964; Bradbury, Chapman, and King 1965*a*, 1965*b*; Bradbury *et al.* 1966; Leeder 1968), and orthocortex and paracortex of Merino wool (Chapman and Bradbury 1968). In this paper a mild method is described for the separation of medullary cells from rabbit, kangaroo, and platypus hair, and results of their amino acid analyses are given.

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II. Experimental Methods

A sample of rabbit kemp hair was obtained from the Division of Textile Physics, CSIRO, Sydney. Hair from the red kangaroo (*Megaleia rufa*) was obtained from the Division of Wildlife Research, CSIRO, Canberra, and fine platypus hair from the Fisheries and Wildlife Department, Melbourne. Porcupine quills from the African porcupine (*Hystrix cristata*) were obtained from the Taronga Park Zoo, Sydney. The samples were cleaned as described previously (Bradbury *et al.* 1966) to remove dirt and grease but no attempt was made to remove the weathered tips of the fibres. Chemicals were either Laboratory or Analytical Reagent grade and were used without further purification.

(*a*) *Separation of Medulla*

(i) *Preliminary Attempts.*—Kangaroo hair was disrupted into its major cellular components by vigorous agitation in 98–100% formic acid at room temperature using a Vibromix agitator (Bradbury *et al.* 1966). The mixture of cortical, cuticle, and medullary cells was separated from the solution by centrifugation, suspended in ethanol, and the bulk of cortical cells removed by a differential screening procedure (Bradbury and Chapman 1964). However, because of the similar sizes of medullary cells, fragments of flat cuticle cells, and a small amount of disrupted cortical cells it was not possible to separate these by a sieving technique. Density-gradient centrifugation in the following liquid systems, viz. ethanol–carbon tetrachloride, ethanol–carbon tetrachloride saturated with sodium acetate or chloral hydrate, aqueous chloral hydrate (Ward and Bartulovich 1956), and aqueous dextran–urographin solution did not produce a separation, hence showing that the density of swollen medulla is very similar to that of swollen cuticle and cortical cells. Separation was not achieved on the basis of a difference in sedimentation velocity due to difference in mass. Attempts at partitioning of the cells in a two-phase aqueous system consisting of 8% (w/w) dextran and 9% (w/w) polyethyleneglycol at pH 6·8 were also unsuccessful (Albertsson 1960). Finally, no separation could be achieved either by passage of the suspension through a short column of glass beads coated with silicone (Shortman 1966) or by attempts at differential flotation of the cellular components (Rubin and Gaden 1962).

(ii) *Final Method.*—The sample of hair was stained with gold by the method of Laxer and Ross (1954). Samples (1 g) of the fibres (cut into short lengths) were disrupted by mechanical agitation in 50 ml of 98–100% formic acid at room temperature for 30-min intervals (Bradbury *et al.* 1966), after which the cellular suspension was separated from the fibres by passage through coarse, stainless steel mesh. The remaining fibres were agitated again in a fresh sample of formic acid for 30 min and the process repeated for total times of not more than 8 hr. Some fibres still remained intact at the conclusion of this treatment. This stepwise procedure has the effect of limiting the time of exposure of cellular components to formic acid and unnecessary mechanical agitation and also gives a preliminary separation, since the early treatments produce mainly cuticle but later ones contain mainly medullary cells. The suspension of cells in ethanol was passed through an 18-μ screen which removed cortical cells (Bradbury and Chapman 1964). The suspension was then layered on a linear carbon tetrachloride–ethanol density gradient (density 1·25–1·45) and was centrifuged in a Servall RC-2 centrifuge at 5°C for 4–8 hr at 1200 *g*. Although the different fibres absorbed different amounts of gold (see tabulation, p. 1207), it was always found that any disrupted cortical cells were found near the top, cuticle near the centre, and medulla near or at the bottom of the tube. The medullary fraction was purified further by a second density-gradient centrifugation, density 1·40–1·50 for rabbit and kangaroo medullae and 1·32–1·45 for platypus medulla. The histological purity of the medulla fraction was always checked by light microscopy (see Fig. 4). The medullary cells were washed with ethanol and water and dried. The yield from the 8-hr agitation treatment was: rabbit 1·8%, kangaroo 2·3%, platypus 1·1%.

(iii) *Aqueous Sodium Hydroxide Method.*—The treatment of Matoltsy (1953) was used for kangaroo hair, but due to the appreciable amount of modification which occurred with rabbit and platypus hair a reduced time of treatment (3 hr) was found to be sufficient to dissolve cuticle and cortical cells. The medullary cells were then collected by centrifugation, washed with water, and dried.

(iv) *Dissection of Porcupine Quills.*—The quill was cut into quarters longitudinally and the pithy interior collected. This medulla was probably contaminated by a small amount of keratinous trabeculae (Rogers 1964).

(*b*) *Microscopy*

The various cellular suspensions were examined routinely using phase-contrast illumination with a Leitz Dialux microscope. A Hardy microtome was used to obtain cross sections of gold-stained fibres for light microscopy. Scanning electron micrographs were obtained with the stereoscan electron microscope located at the Cambridge Instrument Company. For electron microscopy of cross sections, gold-stained fibres were embedded in Araldite, sectioned by means of a Porter–Blum MT 2 microtome fitted with a diamond knife, and examined with a Siemens Elmiskop electron microscope.

(*c*) *Analyses*

The ash content of fibres and cellular components was determined by the Australian Microanalytical Laboratory, Melbourne. Amino acid analyses were performed essentially as described previously (Bradbury, Chapman, and King 1965*a*).

III. Results and Discussion

(*a*) *Location of Gold*

In unstained fibres (Fig. 1) the medulla is seen clearly as the light-coloured, central core, whereas in gold-stained fibres (Fig. 2) the gold is concentrated in the medulla, there being a gradation from the light pink of the cortex (Chapman and Bradbury 1968) to the dark red of the central core. Figure 3 shows the distribution of gold in the cuticle, cortex, melanin granules, and in the larger polyhedral particles up to 100 nm in diameter located in the open structure of the medulla. Ash analyses of the separated histological components of gold-stained kangaroo, rabbit, and platypus fibres show that the medulla contains much more gold than the other components, as may be seen in the following tabulation:

	Ash Content (%)				
	Virgin Fibre	Stained Fibre	Medulla	Cortical Cells	Cuticle
Kangaroo	0·77	11·1	25·3	9·4	10·7
Rabbit	1·28	14·8	24·0	—	—
Platypus	1·10	8·0	14·0	7·6	—

The ash content of the medulla of gold-stained porcupine quill was 15·9%.

The question of the valence state of the gold and its mode of attachment to the protein is still largely unanswered. We have found that the formic acid treatment is an essential part of the staining process and also that some cystine is oxidized to cysteic acid (Chapman and Bradbury 1968). When auric ions are added to solutions of amino acids, coloured complexes are formed at various pH values. Chelation appears to involve primary amino, primary amide, or $^{+}NH_3CHR\,COO^{-}$ groups, or all three, and not simply carboxyl groups as in poly-L-glutamic acid. The preference of gold for amino rather than carboxyl groups is not surprising, because

of the known relative affinities of ligand atoms for acceptor ions (Ahrland, Chatt, and Davies 1958).

(*b*) *Microscopy of Medulla*

The medullary cells produced by disruption of the fibres in formic acid have a characteristic appearance as shown in Figures 4–6. The light micrograph shows the appearance of gold-stained single cells seen from both side-on and end-on views and the way in which they are joined together to form a continuous core for the fibre. This is shown at higher magnification in Figures 5 and 6 and gives a more detailed picture of the exact shape of the intact cell.

It is well known that the medulla contains vacuoles (Ryder 1963; Rogers 1964; Ballin and Happey 1965) and this is confirmed by Figure 3 and also the porous nature of the surfaces shown in Figures 5 and 6. However, it is possible that some of the spaces are produced by extraction of protein material from the medulla during its preparation, which involves immersion in formic acid for about 8 hr. Thus, immersion of porcupine medulla, kangaroo fibres, and Merino wool (which contains no medulla) in formic acid for 9 hr causes dissolution of 14, 4·5, and 1·4% respectively.

Figure 7 shows a cortical cell from platypus hair containing trabeculae which interdigitate between the medullary cells. A finger-like structure occurs in some cases. Cortical cells of this type are also observed with kangaroo hair and had previously been observed in hair from the Australian marsupial *Trichosurus vulpecula* by Chapman (1967) along with cortical cells of the usual shape and size.

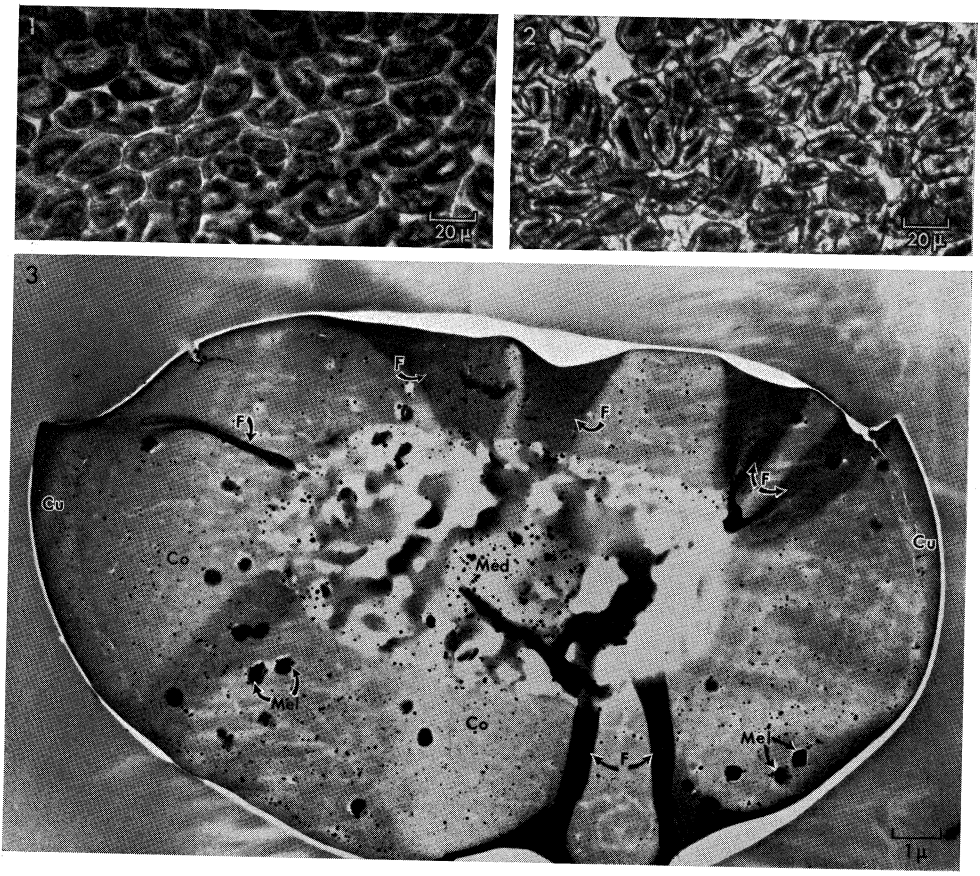
(*c*) *Modification of Medulla during Preparation*

The possibility of some modification of the medulla during its preparation, which involves a three-step procedure using auric ion and formic acid followed by disruption in formic acid at room temperature for 8 hr, was investigated by treatments made on virgin fibres and also on medulla prepared by dissection of porcupine quills. It was shown previously by Chapman and Bradbury (1968) that staining with gold did not change the amino acid composition of Merino wool except for oxidation of part of the cystine to cysteic acid by auric ion. This has now been confirmed for rabbit, kangaroo, and platypus hair and the results for rabbit hair are given in Table 1. Differences of greater than 10% are noted for citrulline and glutamic acid, but these are not reproduced in the analyses for kangaroo and platypus hair or Merino wool and hence must be discounted as due to experimental error. The amount of material extracted from kangaroo fibre by immersion in formic acid for 9 hr is 4·5%, indicating rather minimal modification of the medulla.

Porcupine medulla has a more open structure than either keratin fibres or medulla produced from the latter and is much more susceptible to dissolution by formic acid. Thus 11, 14, and 41% of porcupine medulla is dissolved by immersion in formic acid for 0·67, 9, and 117 hr. The material extracted by formic acid contains relatively more glycine, tyrosine, phenylalanine, and lysine and less ½cystine+cysteic acid and citrulline+ornithine than the medulla. The porcupine medulla is thus slightly altered in amino acid composition by the separation technique, but, because of its more compact structure, the effect on medullae of keratin fibres will be less and can probably be neglected.

(*d*) *Amino Acid Analysis of Medulla*

The results of the amino acid analyses of gold-stained medullary cells of rabbit, kangaroo, and platypus fibres are given in Table 2 together with the values obtained for medulla dissected from porcupine quills. A direct comparison is possible between the results of Rogers (1962) and those obtained in the present work for medulla



Figs. 1 and 2.—Cross sections observed by light microscopy of virgin kangaroo hair under phase contrast (Fig. 1) and kangaroo fibres stained with gold (Fig. 2). Note the concentration of dark melanin granules in the cortex in Figure 1 (see also Fig. 3) and of gold in the central core of the medulla in Figure 2.

Fig. 3.—Electron micrograph of cross section of gold-stained kangaroo fibre. The gold is seen as electron-dense particles in the cuticle (*Cu*), melanin granules (*Mel*), cortex (*Co*), and larger polyhedral particles in the medulla (*Med*). The open structure of the medulla (see also Fig. 5) is clearly shown. Folds produced by the sectioning technique are also shown at *F*.

obtained from the quills of the African porcupine (columns 5 and 6, Table 2). The agreement between the analyses is reasonable except for proline, citrulline+ornithine, phenylalanine, and valine.

Of more importance is an examination of the analyses of medulla in columns 2–5 of Table 2. There is reasonable agreement for all amino acids except tyrosine, $\frac{1}{2}$cystine+cysteic acid, methionine, proline, and lysine in that order, which show

deviations from the mean value in excess of 20%. Because of the absence of any pattern in the differences which do exist they have been averaged to give an analysis (column 7, Table 2) which is regarded as typical of medullary protein. The analyses averaged are of medullae obtained from the hair of a mammal, a marsupial, and a monotreme and from the quill of a mammal. Although it is realized that amino

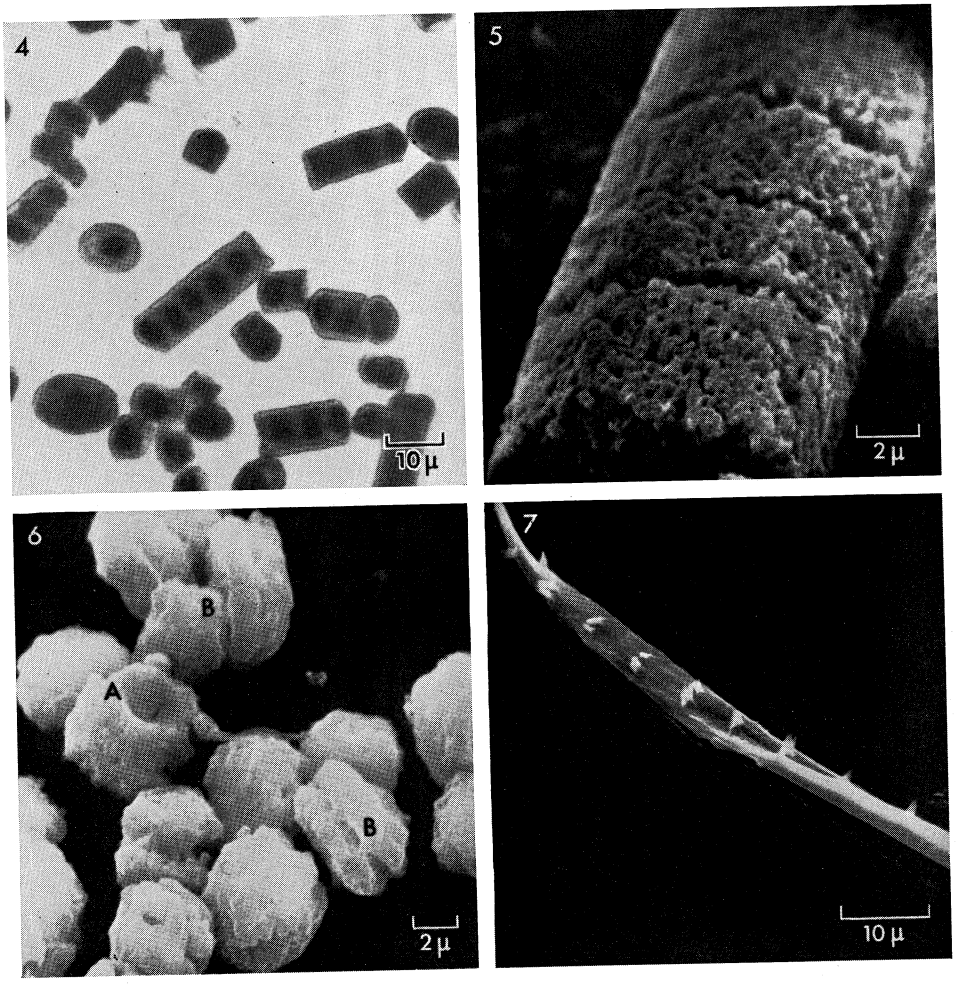


Fig. 4.—Light micrograph of gold-stained medulla from kangaroo hair.

Fig. 5.—Scanning electron micrograph of medullary cells from kangaroo hair stained with gold and coated with a 30-nm-thick layer of gold–palladium prior to examination. Note the porous appearance of the surface and the transverse cracks which separate each cell from its neighbour.

Fig. 6.—Scanning electron micrograph of platypus medulla stained with gold and coated with gold–palladium as in Figure 5. The indentation shown clearly at *A* fits into the rounded end of another cell to form a connected, continuous medulla as shown in Figure 5. Note also the transverse ridges observed at *B*.

Fig. 7.—Scanning electron micrograph of cortical cells from platypus hair coated with gold–palladium as in Figure 5. The trabeculae are clearly visible on one side of the cortical cell and serve to separate the medullary cells and hold them in place.

acid analyses are rather insensitive measures of change in protein constitution, it is rather surprising that medullary samples obtained from such widely divergent sources have such similar amino acid analyses.

It has already been observed by Blackburn (1948) and Rogers (1962) that the medullary protein is very different in composition from α-keratin. If one compares the mean medulla analysis with the mean of the analyses of the three fibres, viz.

TABLE 1

COMPARISON OF AMINO ACID ANALYSES OF VIRGIN AND GOLD-STAINED RABBIT FIBRES AND PORCUPINE MEDULLA

Results given as moles per 100 moles total amino acids

Amino Acid	Rabbit Hair		Porcupine Medulla		
	Virgin	Stained	Unstained	Stained	Formic Acid Extract*
Alanine	4·80	4·86	5·49	5·17	4·43
Arginine	6·55	6·71	4·45	5·35	6·19
Aspartic acid	5·03	5·55	6·76	6·14	5·36
Citrulline	1·01	0·78	8·74	9·70	6·79
Ornithine†	—	—	3·24	5·58	3·66
Citrulline+ornithine	1·01	0·78	11·98	15·28	10·45
Cysteic acid‡	1·18	3·83	0·05	1·86	—
½Cystine	13·36	9·07	1·49	0·68	0·48
Cysteic acid+½cystine	14·54	13·53	1·54	2·54	0·48
Glutamic acid	12·54	14·46	24·42	25·90	22·20
Glycine	8·09	7·75	6·55	5·40	11·90
Histidine	1·56	1·53	1·06	1·12	1·70
Isoleucine	2·79	2·69	2·16	2·00	1·66
Leucine	7·20	6·86	8·97	8·67	8·17
Lysine	2·56	2·77	3·46	2·80	4·10
Methionine	0·78	0·78	0·56	0·43	0·66
Phenylalanine	2·45	2·51	3·31	3·09	4·28
Proline	7·92	7·54	5·36	4·43	4·41
Serine	10·33	10·35	5·13	4·43	4·10
Threonine	6·63	6·23	2·87	2·48	2·17
Tyrosine	2·12	1·94	3·01	2·04	5·38
Valine	3·03	3·15	2·90	2·73	2·34
Recovery of anhydro-amino acids (%)	91	85	97	86	61

* 11% extracted in 0·67 hr at room temperature.

† Produced from citrulline during acid hydrolysis (Rogers 1964; Bradbury and King 1967).

‡ Cysteic acid is produced by oxidation of cystine by means of auric ion which is reduced to gold (Chapman and Bradbury 1968).

rabbit, kangaroo, and platypus (Bradbury *et al.*, unpublished data) then the medulla is rich in glutamic acid, citrulline, leucine, and lysine and poor in cystine, arginine, tyrosine, valine, glycine, proline, serine, and threonine. The medulla is much more extreme in its amino acid composition as compared with the fibre than any other protein histological components isolated thus far from fibres. Some idea may be given of its composition by the statement that about 1 residue in 4–5 is glutamic acid, 1 in 9 is citrulline, 1 in 12 is leucine, 1 in 14 is glycine, and only 1 in 35 is cystine.

(e) *Medulla Produced by Sodium Hydroxide Method*

The usual method for the isolation of medullary cells from hair has been immersion in a concentrated aqueous solution of sodium hydroxide, which dissolves the keratin and leaves medullary cells behind (Blackburn 1948; Matoltsy 1953; Ross 1955; Rogers 1962, 1964). Treatment of a suspension of cortical, cuticle,

TABLE 2

AMINO ACID ANALYSES OF GOLD-STAINED MEDULLARY CELLS OF RABBIT, KANGAROO, AND PLATYPUS FIBRES AND OF MEDULLA DISSECTED FROM PORCUPINE QUILLS

Results given as moles per 100 moles total amino acids

Amino Acid	Rabbit Hair	Kangaroo Hair	Platypus Hair	Porcupine Quill		Mean of Columns 2–5
				This Paper	Rogers (1962)	
Alanine	5·65	5·60	4·91	5·49	5·70	5·48
Arginine	4·35	3·17	3·27	4·45	4·33	3·93
Aspartic acid	5·64	7·57	7·12	6·76	6·75	6·83
Citrulline	6·26	6·85	9·56	8·74	4·43	7·83
Ornithine*	3·41	3·77	4·25	3·24	‡	3·67
Citrulline + ornithine	9·67	10·62	13·81	11·98	‡	11·50
Cysteic acid	3·56†	1·15†	0·91†	0·05	—	—
½Cystine	2·76	0·25	1·26	1·49	1·21	—
Cysteic acid + ½cystine	6·32	1·40	2·17	1·54	1·21	2·88
Glutamic acid	19·41	23·85	26·03	24·42	25·18	22·94
Glycine	6·50	7·45	7·05	6·55	7·73	7·07
Histidine	1·21	1·26	1·43	1·06	1·06	1·25
Isoleucine	2·62	3·05	2·14	2·16	2·34	2·49
Leucine	8·94	8·38	8·05	8·97	9·03	8·55
Lysine	4·91	6·11	5·31	3·46	6·89§	4·97
Methionine	0·91	0·20	0·76	0·56	0·42	0·61
Phenylalanine	2·50	2·61	2·09	3·31	5·87	2·65
Proline	6·15	4·09	3·37	5·36	1·92	4·65
Serine	7·64	6·59	5·51	5·13	5·28	6·29
Threonine	3·85	3·48	3·35	2·87	3·04	3·44
Tyrosine	1·15	0·43	1·61	3·01	4·01	1·58
Valine	2·59	4·14	2·01	2·90	4·81	2·93
Recovery of anhydro-amino acids (%)	75	59	50	97	—	—

* Produced from citrulline during acid hydrolysis (Rogers 1964; Bradbury and King 1967).

† Formed by oxidation of cystine by auric ion which is reduced to gold (Chapman and Bradbury 1968).

‡ Not measured.

§ Value is (lysine + ornithine) since these amino acids were not resolved.

and medullary cells (produced from kangaroo hair by disruption in formic acid) with 3N NaOH, caused dissolution of cortical cells in 12 min and cuticle in 55 min, but the medulla although obviously modified had not dissolved after 24 hr. On the other hand medulla dissected from porcupine quill dissolved to the extent of 79 and 100% in 1 and 2 hr respectively in 3N NaOH. This shows that porcupine medulla is more

readily dissolved in sodium hydroxide than the more compact medullary cells from hairs. A similar effect was observed with formic acid (see above). Of more significance is the fact that the alkali treatment is degradative and is likely to cause major changes in the protein of the medulla.

TABLE 3

AMINO ACID ANALYSES OF KANGAROO MEDULLA AND OF FRACTIONS PRODUCED BY SODIUM HYDROXIDE TREATMENT

Results given as moles per 100 moles total amino acids

Amino Acid	Gold-stained Medulla	Medulla Soluble in NaOH*	Residue after NaOH Treatment*	Medulla Produced from Fibre by NaOH*
Alanine	5·60	6·82	4·12	4·14
Arginine	3·17	4·09	1·24	0·68
Aspartic acid	7·57	8·41	6·07	5·52
Citrulline	6·85	0·86	10·62	15·19
Ornithine	3·77	1·07	5·62	5·91
Citrulline+ornithine	10·62	1·93	16·24	21·10
Cysteic acid	1·15	2·46	0·25	0·00
½Cystine	0·25	1·86	0·79	0·00
Cysteic acid+½cystine	1·40	4·32	1·04	0·00
Glutamic acid	22·85	13·38	38·28	35·42
Glycine	7·45	12·11	3·96	3·13
Histidine	1·26	1·36	1·02	1·03
Isoleucine	3·05	4·09	1·61	1·40
Leucine	8·38	8·70	8·84	8·50
Lysine	6·11	4·82	6·78	6·51
Methionine	0·20	0·19	0·00	0·00
Phenylalanine	2·61	3·23	2·03	1·98
Proline	4·09	4·92	0·00	1·52
Serine	6·59	9·17	3·48	2·71
Threonine	3·48	5·25	1·98	1·43
Tyrosine	0·43	1·73	0·00	2·07
Valine	4·14	5·49	3·31	2·88
Recovery of anhydro-amino acids (%)	59	42	86	46

* Treatment due to Matoltsy (1953); see text.

A detailed study was made by treating gold-stained medulla from kangaroo hair with 3N NaOH for 18 hr, when 43% dissolved. This solution was neutralized, evaporated to dryness, and the residue hydrolysed with hydrochloric acid and analysed for amino acids in the usual way. The residue was washed thoroughly with water, dried, weighed, and analysed. The results are given in Table 3. It is clear that the treatment with sodium hydroxide extracts protein material which, compared with the original medulla, is poor in glutamic acid and citrulline+ornithine and rich in ½cystine+cysteic acid, glycine, alanine, arginine, isoleucine, phenylalanine, proline, serine, threonine, tyrosine, and valine. There is also the likelihood of modification of amino acids present in the polypeptide chain, due to treatment with

strong alkali (Hill 1965), although the amino acid analyses do not show any appreciable effect due to this cause.

A comparison between the amino acid analyses of the medullary residue after alkali treatment and medulla produced from kangaroo hair by the alkali treatment (Matoltsy 1953) is given in columns 4 and 5 of Table 3. There is reasonable agreement between the results for all amino acids except arginine, citrulline+ornithine, cysteic acid+$\frac{1}{2}$cystine, glycine, proline, serine, and tyrosine, which in any case show the correct trend as compared with the result for medulla stained with gold. Comparison of the amino acid analyses of medulla produced by the alkali method from rabbit and platypus hair with the analysis of the corresponding medulla produced by gold staining confirms the assessment made with kangaroo medulla.

It is concluded that the production of medulla by treatment of hairs with strong alkali causes dissolution of a protein from the medulla which is especially rich in glycine and $\frac{1}{2}$cystine+cysteic acid and especially poor in glutamic acid and citrulline+ornithine. The residue remaining is thus very different in composition from the native medulla and hence the alkali method is not to be recommended for the preparation of medulla.

IV. Acknowledgments

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