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High-sulphur Proteins in Mammalian Keratins: a Possible Aid in Classification

Robert C. Marshall, Maurice J. Frenkel and J. M. Gillespie

Division of Protein Chemistry, CSIRO, Parkville, Melbourne, Vic. 3052.

Abstract

A study has been made of the high-sulphur proteins isolated from keratins to determine the value of using their electrophoretic patterns as an aid in studying the classification of closely related animals. The high-sulphur proteins of 35 different species covering nine orders of the class Mammalia have been examined after electrophoresis in polyacrylamide gels at pH 2·6. From this study it can be concluded that keratin samples which have different electrophoretic patterns can be tentatively judged as coming from animals of different species, although identical patterns do not necessarily mean identity of animal source. The ease of keratin sampling and of electrophoretic analysis could make the method useful as an aid in classification.

Introduction

Animal hairs are normally identified by microscopic examination (Appleyard 1960; Brunner and Coman 1974), a technique not without its difficulties because of the variety of the hair types produced by a single animal, because of structural variations along a single fibre, and because dissimilar animals may have hairs with similar structure. In spite of recent developments in this field (Brunner and Coman 1974), in many cases the identification of hairs still requires the subjective assessment of colour, crimp and pigment granule characteristics. There is clearly room for an independent method to complement the microscopic technique.

In a comparative study of the high-sulphur protein fraction isolated from sheep wool and goat hair, Darskus and Gillespie (1970, 1971) observed major differences in the electrophoretic banding patterns. This enabled these animals, which are difficult to distinguish biochemically (Curtain 1971), to be clearly differentiated. It was suggested by Lindley *et al.* (1971) that electrophoresis of other hair proteins might be a useful tool for studying taxonomic problems and for the identification of hairs. Although an electrophoretic procedure may seem considerably more complicated than the microscopic method, it should be pointed out that a comparable electrophoretic procedure is now used in the routine identification of wheat varieties at regional centres in Australia during harvest (Wrigley and Shepherd 1974; Wrigley and McCausland 1975).

Hair, being the most accessible mammalian tissue, can be sampled easily from most animals without injury. The fact that all fibres from one animal contain the same proteins simplifies sampling, and even naturally shed hair can be used although best results are obtained with samples clipped close to the skin (Darskus and Gillespie 1971). Hair is naturally fairly resistant to chemical and biological attack and thus

the range of experimental material extends to museum specimens, fossil material (Gillespie 1970), and possibly even to fibres from faeces and coprolites.

Darskus and Gillespie (1970, 1971) carried out their investigation using electrophoresis at acid pH on starch gel, a technique which did not successfully resolve the high-sulphur proteins of other animal hairs. Day (1972) attempted to relate variation in electrophoretic patterns of keratin protein to morphological differences. Although some success was achieved, there were a number of unexplained deviations from a linear correlation. These may have arisen from the techniques employed. The electrophoretic procedure used by Darskus and Gillespie (1971) has recently been modified (Marshall and Gillespie 1976*a*, 1976*b*) and has made feasible an extensive study of the high-sulphur proteins from a range of animal hairs sufficiently wide to illustrate the usefulness and the limitations of the technique.

The work presented in this paper is designed to critically examine a number of questions related to the use of keratin high-sulphur proteins for investigating the taxonomy of mammals. The study attempts to determine whether the electrophoretic patterns of these proteins are species-specific, i.e. whether each species has a unique pattern; if there is a progressive change in the degree of similarity when the high-sulphur proteins from different species, genera, families and orders are compared; and if each order has characteristic protein bands which can be observed in the hair of animals of that order.

Experimental and Methods

Origin and Preparation of Keratin Samples

Sources of keratins are given at the end of this paper. The generic and common names of the animals from which keratin samples were obtained are shown in Table 1. Keratins were cleaned by solvent and water extraction (Gillespie and Inglis 1965).

Preparation of High-sulphur Proteins

All mammalian keratins contain three main protein fractions, descriptively termed low-sulphur, high-sulphur and high-tyrosine proteins (Gillespie and Frenkel 1974). The present work has been carried out using only the high-sulphur protein fraction. The low-sulphur proteins, although constituting the major proportion of most keratins, contain a relatively small number of electrophoretic components, which do not appear to differ greatly between animals (Sparrow, personal communication). The high-tyrosine proteins, which appear to differ in electrophoretic pattern for at least a small number of species (Gillespie 1972), constitute less than 1% of some keratins (Gillespie and and Frenkel 1974). High-sulphur proteins, which generally account for more than 15% of the proteins of keratins (Gillespie and Frenkel 1974) have been observed to differ greatly in their electrophoretic patterns for a number of different animals (Lindley *et al.* 1971).

High-sulphur proteins were isolated from keratins after solubilization of the keratin by alkaline reduction in urea, alkylation with iodoacetate and dialysis against deionized water (Gillespie and Reis 1966). Addition of zinc acetate to 0·02M (pH 6·0) to this dialysate precipitates the low-sulphur and high-tyrosine proteins which are sedimented by centrifugation, leaving the high-sulphur components in solution. This supernatant is made 0·02M in sodium citrate, dialysed against deionized water and freeze-dried.

In cases where hair was not available from a particular species, high-sulphur protein was extracted from another keratin type (porcupine and North American porcupine quills, rhinoceros horn). High-sulphur proteins isolated from different keratin types of the one species have been shown (Lindley *et al.* 1971; Marshall and Gillespie, unpublished results) to contain the same components, although their relative proportions may vary slightly.

Table 1. Taxonomic classification and common names of animals from which keratin samples were obtained

Classification follows that of Morris (1965), except for some members of the genus *Macropus*, classified according to Poole

Order	Family	Genus and species	Common name
Monotremata	Tachyglossidae	*Tachyglossus aculeatus*	Echidna
Marsupialia	Phalangeridae	*Trichosurus vulpecula*	Brush-tailed possum
	Macropodidae	*Macropus giganteus*[A]	Eastern grey kangaroo
		M. fuliginosus[A]	Western grey kangaroo
		M. rufus[B]	Red kangaroo
		M. robustus robustus[A]	Wallaroo
		M. r. erubescens[A]	Euro
		Protemnodon rufogrisea	Red-necked wallaby
		P. bicolor	Swamp wallaby
		P. eugenii	Kangaroo I. wallaby
	Vombatidae	*Vombatus ursinus*	Common wombat
Primates	Hominidae	*Homo sapiens*	Man
	Cercopithecidae	*Macaca irus*	Monkey
Lagomorpha	Leporidae	*Oryctolagus cuniculus*	Rabbit
Rodentia			
Myomorpha	Muridae	*Mus musculus*	Mouse
		Rattus rattus	Rat
	Cricetidae	*Cricetus cricetus*	Hamster
Hystricomorpha	Caviidae	*Cavia* sp.	Guinea-pig
	Erethizontidae	*Erethizon dorsatum*	N. American porcupine
	Hystricidae	*Histrix cristata*	Porcupine
Carnivora	Canidae	*Vulpes vulpes*	Fox
		Canis familiaris	Dog
	Felidae	*Felis catus*	Cat
Proboscidea	Elephantidae	*Elephas maximus*	Elephant
Perissodactyla	Rhinocerotidae	*Diceros bicornis*	Rhinoceros
Artiodactyla			
Tylopoda	Camelidae	*Camelus dromedarius*	Camel
		Lama pacos	Alpaca
		L. glama	Llama
Ruminantia	Bovidae		
	Caprinae	*Ovibos moschatus*	Musk ox
		Hemitragus jemlahicus	Himalayan tahr
		Ovis aries	Merino sheep
		Capra hircus	Angora goat
	Bovinae	*Tragelaphus spekei*	Sitatunga antelope
		Bos grunniens	Yak
		B. taurus	Jersey cattle

[A] Classified according to W. E. Poole, Division of Wildlife Research, CSIRO.
[B] More recently as *Megaleia rufa* (e.g. Ride 1970).

Polyacrylamide Gel Electrophoresis

The proteins were examined by electrophoresis in 10% polyacrylamide slab gels (acrylamide : bis-acrylamide 27 : 1) containing 4·8M acetic acid and 2·75M urea at pH 2·6 for 2 h at 250 V. The gels were stained with Coomassie blue. Full details of the technique and equipment used are given by Marshall and Gillespie (1976*a*). Each slab gel run included a sample of high-sulphur protein from Merino wool as a reference.

Results

Comparison of Electrophoretic Profiles

(i) *Between orders*

Nine samples of the high-sulphur fraction, each from a keratin representing, but not necessarily representative of, a different order, were examined by electrophoresis on polyacrylamide at pH 2·6. It can be seen (Fig. 1) that each sample is heterogeneous, the sheep pattern containing the greatest number of resolved bands. However, even the relatively simple patterns have a strong background stain which indicates extreme heterogeneity.

Apart from the high-sulphur proteins of rhinoceros horn and wool, each electrophoretic pattern in Fig. 1 spans almost the same distance on the gel. Such a situation might be expected in a set of homologous proteins which cover approximately the same range of size and charge. Although each pattern in Fig. 1 contains a unique set of bands, it can be seen that certain pairs of unrelated animals e.g. rabbit and rat, or kangaroo and monkey, have high-sulphur protein patterns which are very similar. This observation would tend to restrict comparisons of animals from different orders on the basis of their keratin high-sulphur protein electrophoretic patterns.

(ii) *Within an order*

Although the value of comparing animals from different orders on the basis of their high-sulphur protein electrophoretic patterns is limited, it is of interest to examine whether within a particular order there are certain characteristic protein bands found in all its members. For this study, orders where animals from two or more genera were available were investigated. These included the orders Marsupialia, Primates, Rodentia, Carnivora and Artiodactyla (Fig. 2).

For three genera of the order Marsupialia there appears to be a common arrangement of electrophoretic bands in which there is a group of major components of relatively low mobility and a group of minor bands, often three, with greater mobility. Comparisons between these genera do reveal small but significant differences in the mobility of components. The wallaby high-sulphur proteins have a slightly greater electrophoretic mobility than those of the kangaroo, and in the possum the three minor components have a greater mobility than in the others. The pattern for the wombat (Fig. 2), the fourth genus examined, does not conform to this common arrangement of bands.

The high-sulphur proteins isolated from primate hairs appear to contain about the same number of components, which span a similar mobility range (Fig. 2). With the possible exception of one band, there are significant differences in both the mobility and staining intensity of comparable components. Since only two primate specimens were examined, it is not possible to speculate on common banding patterns for this order.

When keratins of Rodentia are examined there appear (Fig. 2) to be no similarities between the electrophoretic patterns for the suborders Myomorpha and Hystricomorpha. Within Myomorpha, mouse, rat, and hamster hair patterns show overall similarity in number and relative intensity of bands although there are significant mobility differences for some components. There does not appear to be a common pattern for the Hystricomorphs examined, and a more extensive study would be required to determine if common features do exist.

The two families of Carnivora show a common distribution of protein components (Fig. 2). There appear to be two distinct groups of bands with a region of ill-defined material between them. Differences in the patterns of the fox and dog are confined to the components of greater mobility; the protein of the cat show bands common to dog or fox but not both.

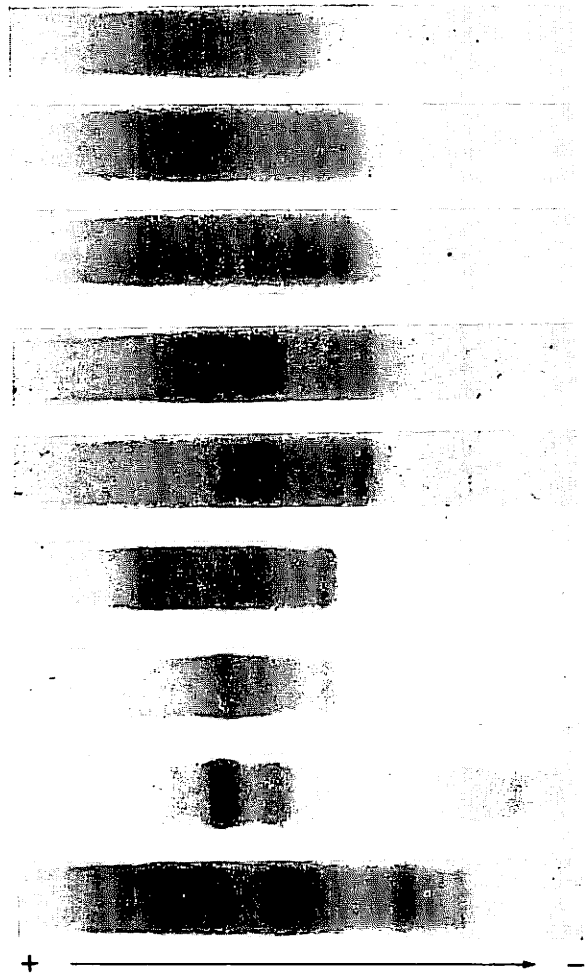

Fig. 1. Polyacrylamide gel electrophoretic patterns of high-sulphur proteins extracted from the keratins of animals from different orders. Common names are shown on the figure.

Within the Artiodactyla, there does not appear to be a common electrophoretic pattern for the suborders Tylopoda and Ruminantia, nor for the subfamilies Bovinae and Caprinae (Fig. 2). In the subfamily Caprinae, the four genera which were examined showed marked differences in their electrophoretic patterns (Fig. 2). Merino sheep wool shows the most complex banding pattern with at least 11 components. The goat protein appears to have seven components in common with wool, but at least two bands, one of which is major, differ significantly in mobility from any wool component. The musk ox and Himalayan tahr have very different patterns, although there may be a few bands with mobilities similar to sheep or goat proteins.

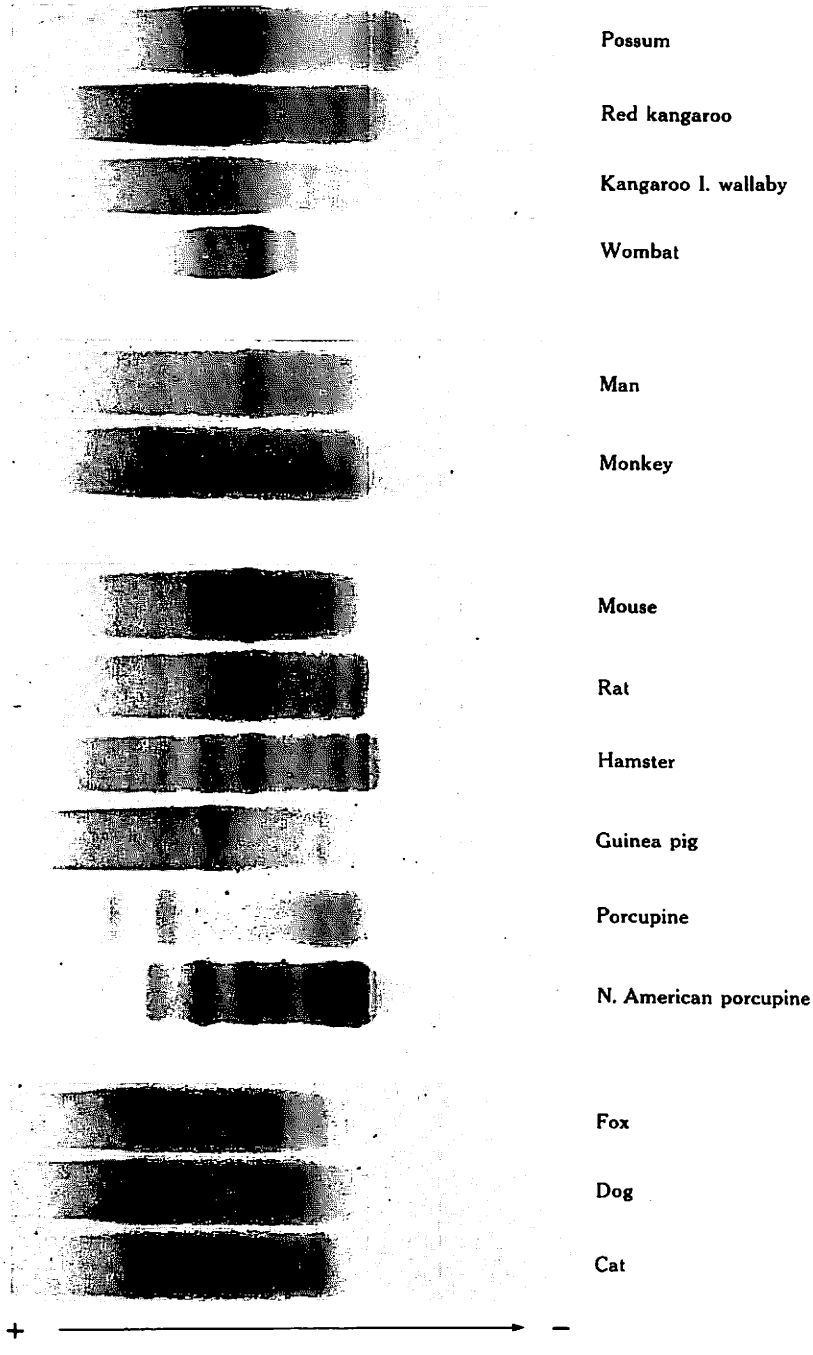

Fig. 2. Polyacrylamide gel electrophoretic patterns of high-sulphur proteins extracted from the keratins of animals from different genera within a number of orders. Common names are shown on the figure.

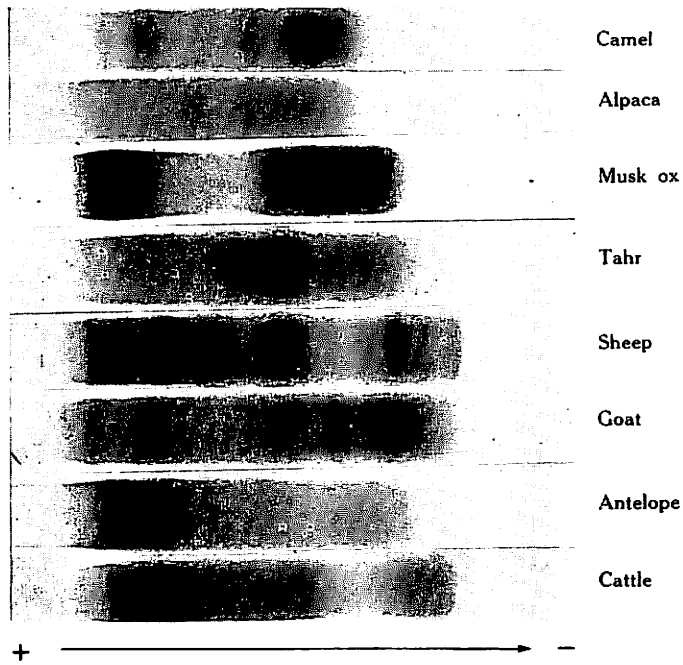

Fig. 2. (*Continued*)

Although the keratin from only one member of the Monotremata has been shown in this study (Fig. 1), it is of interest to note that the proteins of platypus hair (unpublished observation) appear to have a similar number and relative mobility to those from the echidna quill. The platypus pattern had a relatively dense background stain and observations were made directly from the polyacrylamide gel.

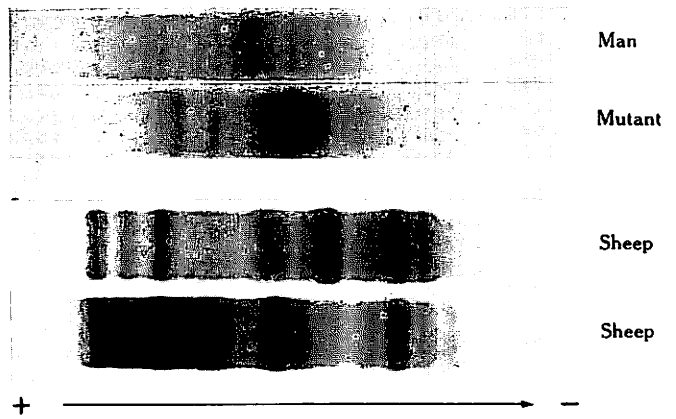

Fig. 3. Polyacrylamide gel electrophoretic patterns of high-sulphur proteins extracted from the keratins of animals from the same species within two genera. Common names are shown on the figure.

(iii) *Within genus*

Different genera within a suborder generally appear to have high-sulphur proteins with different electrophoretic patterns. For example, rat and mouse, and camel and

alpaca, show significant, although not large, differences (Fig. 2). When different species of a single genus are examined the patterns may be very similar or quite different (Fig. 4). Within the genus *Macropus*, the patterns for the eastern and western grey kangaroos are identical. However, the grey and red kangaroos show marked differences throughout their electrophoretic patterns. Within this genus, the wallaroo

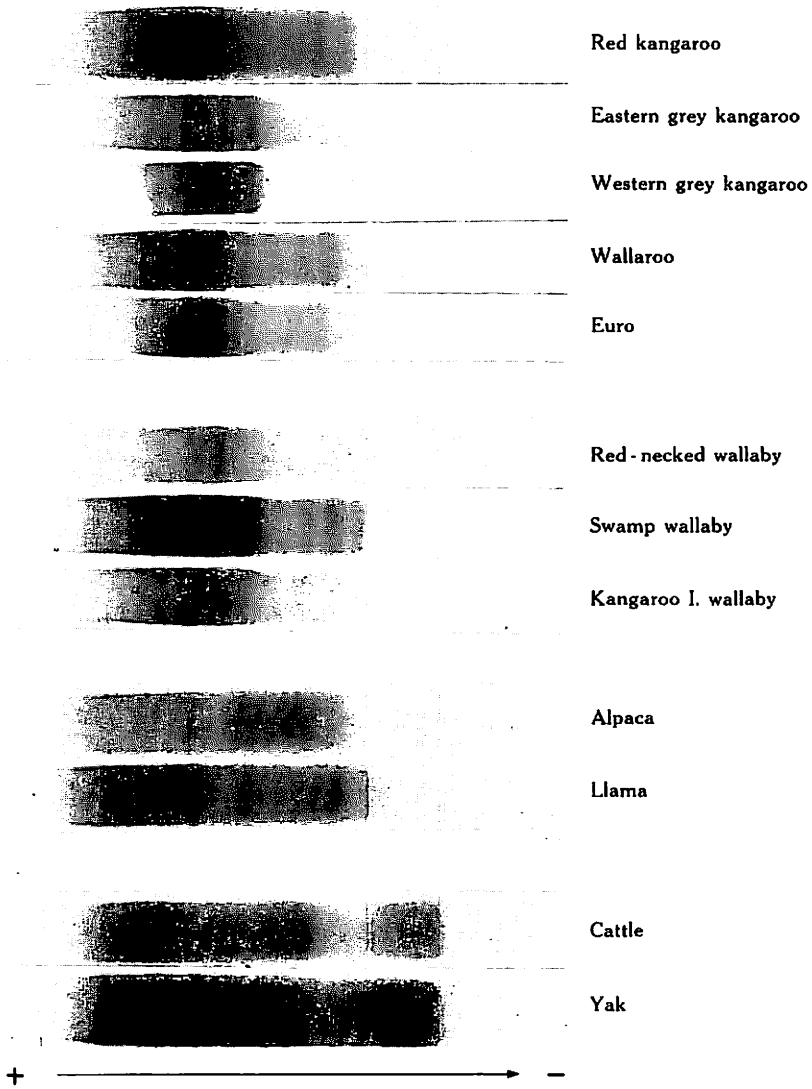

Fig. 4. Polyacrylamide gel electrophoretic patterns of high-sulphur proteins extracted from the keratins of animals from different species within a number of genera. Common names are shown on the figure.

and euro have an identical pattern to the red kangaroo, apart from some minor differences in intensity. The two species of the genus *Bos*, yak and Jersey cattle, have a similar number of components, but there are significant differences in the mobilities of many of them. A similar situation exists for the alpaca and llama, both species

of the genus *Lama* (Fig. 4). On the other hand, when three species of the genus *Protemnodon* are compared (Fig. 4), the patterns are identical.

(iv) *Within a species*

Individuals from particular species were examined to determine the degree, if any, of variation in the electrophoretic patterns of their high-sulphur proteins. Samples of seven red kangaroos and seven grey kangaroos give indistinguishable patterns for individuals within a species. Similarly, proteins from seven mice, including three mutants (two tabby (TA^+ ♀, TA° ♂) and one naked (N) mutants) reveal no discernible differences.

In contrast to the above observations, a sample of mutant human hair (Pollit and Stonier 1971) produces a distinctly different electrophoretic pattern to a normal control (Fig. 3). Differences can also be observed between the patterns of two fine non-Peppin Merino sheep housed and fed under identical conditions (Fig. 3). The patterns are very similar, but the component with greatest mobility in one sample is missing from the other, and there are also small variations in the relative proportions of other protein bands.

Discussion

In the present study an attempt has been made to extend the work of Darskus and Gillespie (1971), which showed that sheep and goats could be readily distinguished by their keratin high-sulphur protein electrophoretic patterns. It was of interest to extend these findings to determine if the high-sulphur proteins are species-specific and if the technique is of value in the classification of closely related animals.

Although many workers have studied proteins isolated from a wide variety of keratins (reviewed by Fraser *et al.* 1972), only Day (1972) has attempted to use the electrophoretic patterns of keratin proteins as an aid in animal classification. He examined whole-protein extracts and used the relative mobilities of the protein bands to calculate coefficients of similarities for pairs of species, and to compare a classification based on these coefficients with one based on morphological characteristics. Only a limited success was achieved and some large deviations from a linear correlation were unexplained. The discrepancies in Day's classification could be partly explained by his use of the whole-protein extract of keratin. The electrophoretic patterns are dominated by a relatively small number of components (mainly low-sulphur proteins) compared with the total number of proteins present in each keratin, and therefore mobility calculations are more or less restricted to these major bands. The majority of components visible in the electrophoretic patterns presented had relative mobilities in the range 0·3–0·5, making precise calculations difficult. Furthermore, recent studies of the low-sulphur proteins (Sparrow, personal communication) suggest that these proteins are very similar for a numberof different animals, although Hrdy and Baden (1973) in a study limited to six primates found that this was not invariably true.

For the electrophoretic technique described in this paper to be of use in animal classification and identification, it is necessary that the proteins from hairs of the same species always have the same electrophoretic patterns, whereas those from hairs of different species always give patterns differing in number or mobility of bands. How far do the results presented in this paper meet these criteria? For a selection

of red and grey kangaroos and normal and mutant mice, electrophoretic patterns of hair proteins within a species were identical. Upwards of 50 sheep examined by ourselves and Darskus and Gillespie (1971) gave identical patterns, apart from small differences in band intensity, although a variant was found that lacks one of the 13 normally observable bands (Fig. 3). A genetic defect in man gave a quite abnormal electrophoretic pattern (Fig. 3), but the difficulties in quantitatively extracting adult hair makes interpretation of this result difficult. The conclusion may be drawn that, in general, animals within a species have recognizably similar patterns.

The situation is not so clear for differences between species of the same genus. Although different species within the genera *Bos* and *Lama* have significantly different patterns (Fig. 4), the three species of genus *Protemnodon* gave identical patterns. Within the genus *Macropus* the pattern for the grey kangaroo differs from that of the red kangaroo and wallaroo. The difference in patterns between the red and grey kangaroos supports the recent classification of these species into separate genera (Frith and Calaby 1969; Ride 1970). On the other hand, the identical patterns found for wallaroo and red kangaroo (Fig. 4), classified by the same workers into different genera, are difficult to explain.

From these results it appears that, although animals of the same species generally have identical electrophoretic patterns, animals from different species within a genus or even from different genera do not invariably have dissimilar patterns. There does not appear to be any gradation in the degree of similarity as animals from different species, genera, families and orders are examined, and only in the case of the Marsupialia is there an indication of a characteristic banding pattern for an order. These observations are somewhat limited, as a result of the relatively small number of animals investigated. Clearly then, the method has less potential for animal classification than was at first thought by Darskus and Gillespie (1970, 1971) and Lindley *et al.* (1971). However, the ease of collection of hair samples in comparison with tissue samples, and the simplicity of the experimental technique, coupled with the high level of specificity observed in certain cases, e.g. in the Artiodactyla, suggest that it may well be of use as a supplementary tool in animal classification. The very high degree of constancy found for the keratins from three species suggests that different patterns for samples can be tentatively regarded as meaning that the animals belong to different species. Unfortunately, identical patterns cannot be taken as proof of identity of species.

It should be pointed out that most bands in the electrophoretic patterns of at least wool and mouse hair (Darskus 1972; Marshall and Gillespie 1976*a*, 1976*b*) contain more than one protein. It is possible therefore that the development of an electrophoretic procedure capable of greater resolution than is now obtainable may well eliminate some of the ambiguities found in the present study.

Although a systematic study has not been reported, sufficient is known about the electrophoretic patterns of feather (Harrop and Woods 1967) and beak proteins (Frenkel and Gillespie, unpublished data) to suggest that the methodology reported here may also be of value in studying taxonomic relationships of birds.

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References

Appleyard, H. M. (1960). Guide to the identification of animal fibres. (Wool Industries Research Association: Leeds.)

Brunner, H., and **Coman, B. J.** (1974). The identification of mammalian hair. (Inkata Press: Melbourne.)

Curtain, C. C. (1971). On the origin of domesticated sheep. *Antiquity* **45**, 303–4.

Darskus, R. L. (1972). Electrophoretic and chromatographic characterization of sulphur-rich proteins from wool. *J. Chromatogr.* 69, 341–8.

Darskus, R. L., and **Gillespie, J. M.** (1970). Comparison of high-sulphur proteins from some animal hairs. *Proc. Aust. Biochem. Soc.* **3**, 56.

Darskus, R. L., and **Gillespie, J. M.** (1971). Breed and species differences in the hair proteins of four genera of Caprini. *Aust. J. Biol. Sci.* **24**, 515–24.

Day, T. H. (1972). Interspecific variation in the hair proteins. *Comp. Biochem. Physiol. B Comp. Biochem.* **43**, 361–7.

Fraser, R. D. B., MacRae, T. P., and **Rogers, G. E.** (1972). 'Keratins.' (Charles C. Thomas: Springfield, Illinois.)

Frith, H. J., and **Calaby, J. H.** (1969). 'Kangaroos.' (F. W. Cheshire: Melbourne.)

Gillespie, J. M. (1970). Mammoth hair: Stability of α-keratin structure and constituent proteins. *Science* (*Wash. D.C.*) **170**, 1100–2.

Gillespie, J. M. (1972). Proteins rich in glycine and tyrosine from keratins. *Comp. Biochem. Physiol. B Comp. Biochem.* **41**, 723–34.

Gillespie, J. M., and **Broad, A.** (1972). Ultra-high-sulphur proteins in the hairs of the Artiodactyla. *Aust. J. Biol. Sci.* **25**, 139–45.

Gillespie, J. M., and **Frenkel, M. J.** (1974). The diversity of keratins. *Comp. Biochem. Physiol. B Comp. Biochem.* **47**, 339–46.

Gillespie, J. M., and **Inglis, A. S.** (1965). A comparative study of high-sulphur proteins from α-keratins. *Comp. Biochem. Physiol.* **15**, 175–85.

Gillespie, J. M., and **Reis, P. J.** (1966). The dietary-regulated biosynthesis of high-sulphur wool proteins. *Biochem. J.* **98**, 669–77.

Harrap, B. S., and **Woods, E. F.** (1967). Species differences in the proteins of feathers. *Comp. Biochem. Physiol.* **20**, 449–60.

Hrdy, D., and **Baden, H. P.** (1973). Biochemical variation of hair keratins in man and non-human primates. *Am. J. Phys. Anthropol.* **39**, 19–24.

Lindley, H., *et al.* (1971). The high-sulphur protein fraction of keratins. Appl. Polym. Symp. No. 18, pp. 21–35.

Marshall, R. C., and **Gillespie, J. M.** (1976*a*). High-sulphur proteins from α-keratins I. Heterogeneity of the proteins from mouse hair. *Aust. J. Biol. Sci.* **29**, 1–10.

Marshall, R. C., and **Gillespie, J. M.** (1976*b*). High-sulphur proteins from α-keratins II. Isolation and partial characterization of purified components from mouse hair. *Aust. J. Biol. Sci.* **29**, 11–20.

Morris, D. (1965). 'The Mammals: A Guide to the Living Species.' (Hodder and Stoughton: London.)

Pollitt, R. J., and **Stonier, P. D.** (1971). Proteins of normal hair and of cystine-deficient hair from mentally retarded siblings. *Biochem. J.* **122**, 433–44.

Ride, W. D. L. (1970). 'A Guide to the Native Mammals of Australia.' (Oxford University Press: Melbourne.)

Wrigley, C. W., and **McCausland, J.** (1975). Instruction manual on laboratory procedures for identifying grain samples of Australian wheat varieties. CSIRO Wheat Res. Unit Tech. Publ. No. 3.
Wrigley, C. W., and **Shepherd, K. W.** (1974). Identification of Australian wheat cultivars by laboratory procedures: examination of pure samples of grain. *Aust. J. Exp. Agric. Anim. Husb.* **14**, 796–804.

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