CHAPTER 8

Peptide mapping of S-carboxymethylated hair and feather proteins using 2D-dimensional electrophoresis

8.1 INTRODUCTION

Although hair and feather proteins have been widely studied, not much work has been done on the peptide mapping of radiolabelled S-carboxymethylated proteins. Hair and feather proteins may be labelled with ^{14}C-iodoacetic acid. The extent of labelling depends largely on the cysteine content (Marshall, 1983).

A convenient method for peptide mapping of separated individual proteins in one-dimension has been described in which the proteins (mostly still within a polyacrylamide matrix) were subjected to limited proteolysis in the presence of SDS and cleavage products analysed by polyacrylamide gel electrophoresis (Cleveland *et al.*, 1977).

This method was adopted for the peptide mapping of heterogeneous protein mixtures using 2D-electrophoresis (Bordier and Crettol-Jarvinen, 1979) and it was claimed that in complex samples, up to 20 individual proteins could be analysed simultaneously. Furthermore, any individual protein consisting of only 1% of the total mixture, generated a peptide pattern that could clearly be identified. A method for peptide mapping of low-sulphur proteins from normal and variant human nail samples using papain digestion has previously been described (Marshall, 1980).

I was interested in developing a technique similar in scope to the previous method (Bordier and Crettol-Jarvinen, 1979) for the simultaneous peptide mapping of a range of human and animal hair proteins as well as the proteins of avian feathers. Feather proteins show a pattern completely different from human and other mammalian hair protein profiles (Khawar *et al.*, submitted for publication). I have also observed a large apparent molecular mass multiplicity in the hair proteins of a range of mammalian species (Khawar *et al.*, submitted for publication), and was concerned to explore possible homology relationships between apparently electrophoretically discrete proteins both within and between species. By staining for protein and performing autoradiography on the same 2D-gels I might identify, in particular, cysteine-rich peptides derived from individual proteins in the complex mixture and therefore possibly address the issue of homology and duplication in the domain structures of various keratins both within and between species.

8.2 RESULTS

8.2.1 Comparison of tryptic cleavage pattern between Coomassie blue stained gel and autoradiograph

Fig. 8.1a shows the tryptic cleavage pattern of human (Australian European of Anglo-Celtic origin) hair proteins stained with Coomasie blue.

Fig. 8.1b shows an autoradiograph of the tryptic cleavage pattern of the same sample showing the incorporation of ^{14}C-iodoacetic acid. The radioactive profile shows some differences from the simple Coomassie blue pattern demonstrating the differential distribution of cysteine containing cleavage fragments. Note that the undigested proteins lie along the diagonal and the tryptic fragments derived from each resolved component below the diagonal. Fig. 8.1(c) shows the radioactive profile for a different human (Australian Aboriginal) sample. Although the profile for the Australian Aboriginal (human) sample (Fig. 8.1c) is very similar to that observed for the Australian European (human) sample (Fig. 8.1b) some differences are marked with arrows in Fig. 8.1(b) and (c).

Fig. 8.2a shows peptide mapping of proteins from goat (Angora) hair stained with Coomassie blue, while Fig. 8.2b shows the autoradiograph of the same sample. Peptide fragments containing cysteine-rich domains are indicated by arrows.

Fig. 8.3a shows trypsin digested chicken feather proteins and Fig. 8.3b shows the autoradiograph of the same sample. Clearly, the chicken proteins were resistant to trypsin digestion under these conditions.

Although the chicken feather proteins showed very little apparent digestion some bands in the very low molecular mass region could be seen after the second dimension separation.

8.2.2 Radioactive peptide pattern as an indication of species relatedness

Fig. 8.4(a, b, c and d) shows the radioactive peptide pattern of proteins from rat, possum, mountain pygmy possum and duck respectively. Differences among species are again evident both in terms of the enhanced separation of the undigested hair and feather proteins along the diagonal by comparison with the simple 1D separation and also in terms of the tryptic digestion profile.

In the profile for rat (Fig. 8.4a) most of the radiolabelled proteins lie undigested on the diagonal toward the lower end of the molecular mass distribution. The proteins in the mid-molecular mass range (a, b on Fig. 8.4a) show considerable digestion with a species specific pattern. There are no high molecular mass radiolabelled proteins as clearly present in both human samples (Fig. 8.1a, b,c).

The profiles for possum (Fig. 8.4b) and mountain pygmy possum (Fig. 8.4c) are similar but discrete. Again most of the digested proteins lie in the mid-molecular mass range (a, b on Fig. 8.4b, c). The pattern for duck (Fig. 8.4d) is most similar to the

pattern seen for chicken (Fig. 8.3b). In this case though there are only four undigested very low molecular mass bands lying on the diagonal in the second dimension. For the duck, some slight tryptic digestion was seen for several of these low molecular mass zones.

It should be emphasised here that the results presented are derived from 1D radioactive gels that had been stored for up to six months prior to rehydration, proteolysis and the separation of the radioactive peptides in the second dimension. Profiles were no different to gels which were freshly run in the 1st dimension without drying and storage (results not shown).

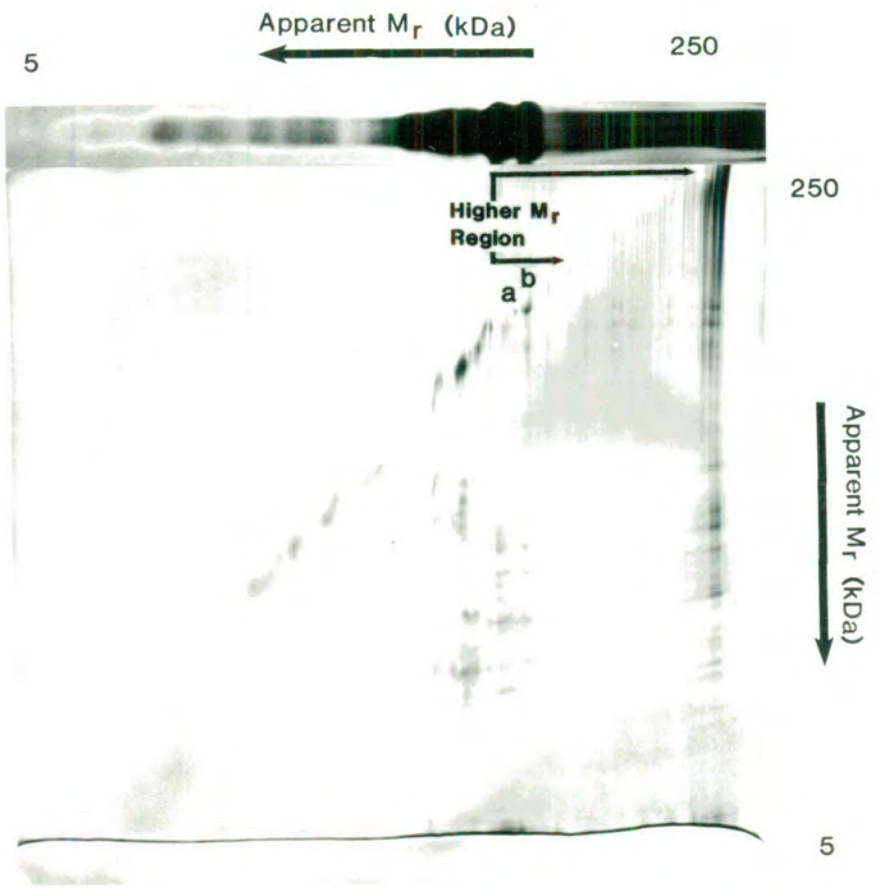

Fig. 8.1(a) Tryptic cleavage pattern of human hair proteins (Australian European of Anglo-Celtic origin). Proteins were S-carboxymethylated with ^{14}C-IAA and the gel stained with Coomassie blue. Mid-range molecular mass protein bands are indicated by a and b.

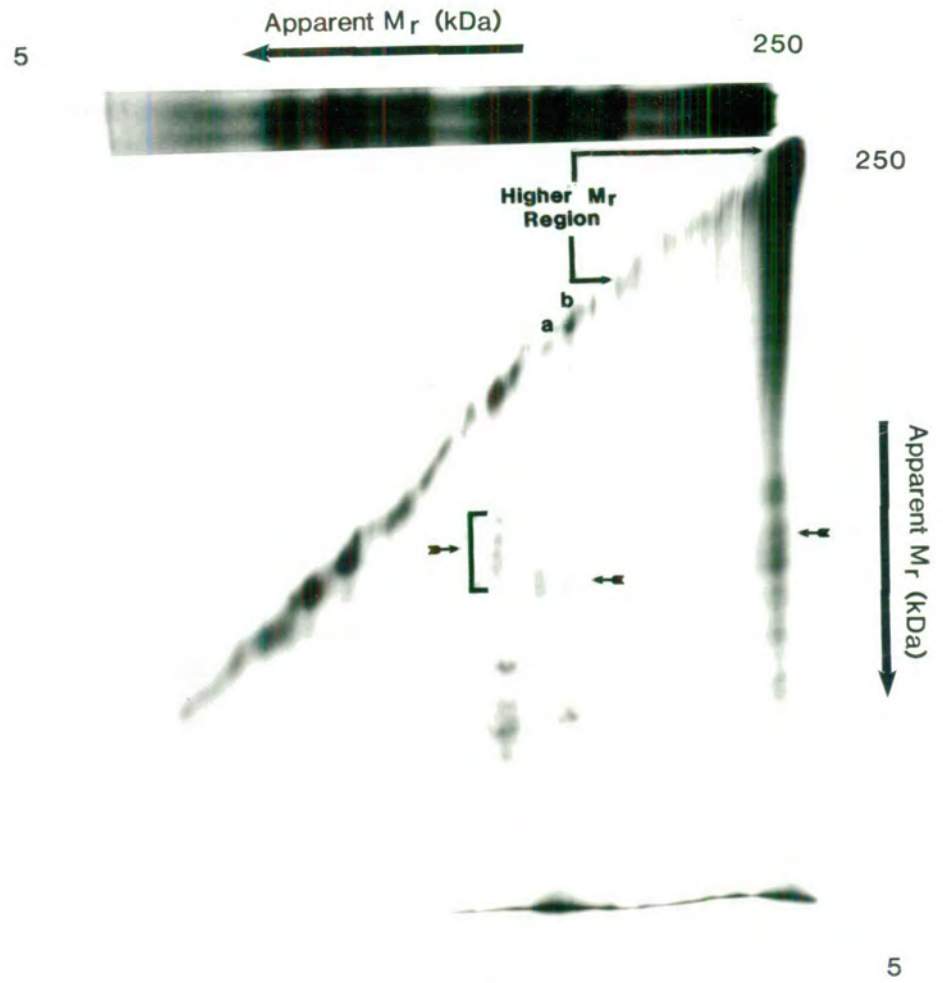

Fig. 8.1(b) Fluorograph of the same sample (Australian European of Anglo-Celtic origin) as represented in Fig. 8.1(a). Mid-range molecular mass protein bands are indicated by a and b. Arrows indicate protein zones which differ from those observed in Australian Aboriginal samples (see Fig. 8.1(c)).

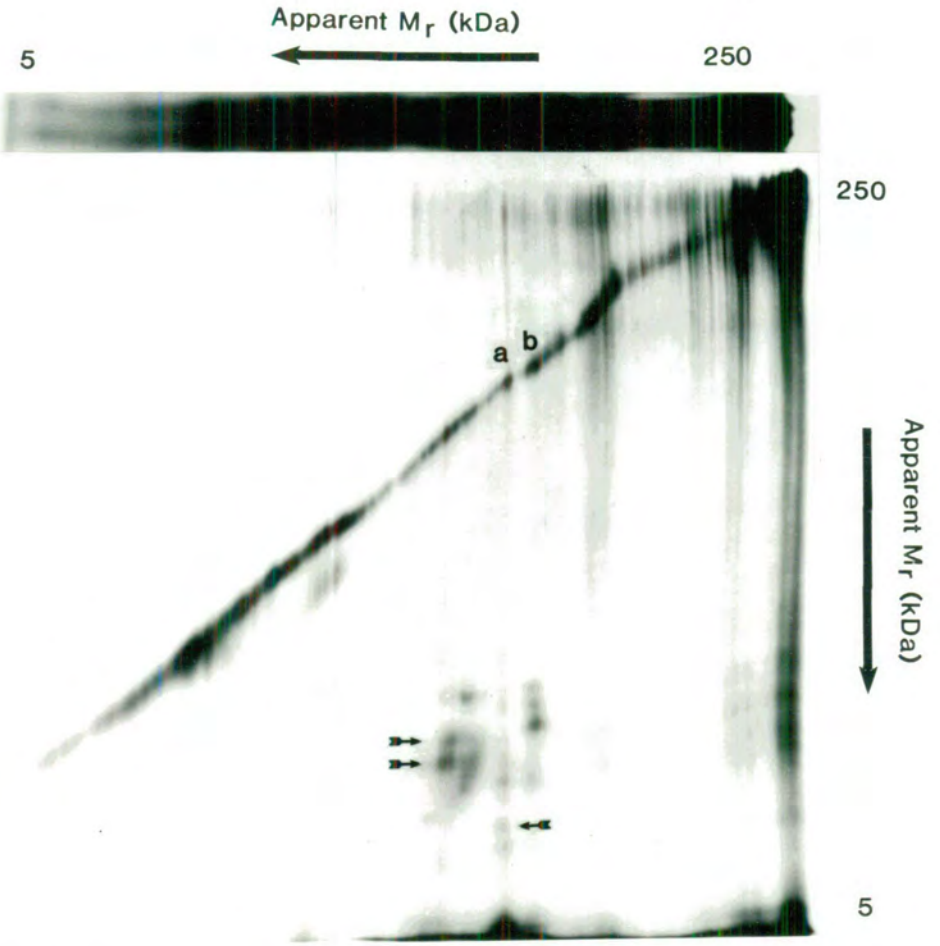

Fig. 8.1(c) Fluorograph of human hair proteins (Australian Aboriginal). Mid-range molecular mass protein bands are indicated by a and b. Arrows indicate protein zones which differ from those observed in Australian European samples (see Fig. 8.1(b)).

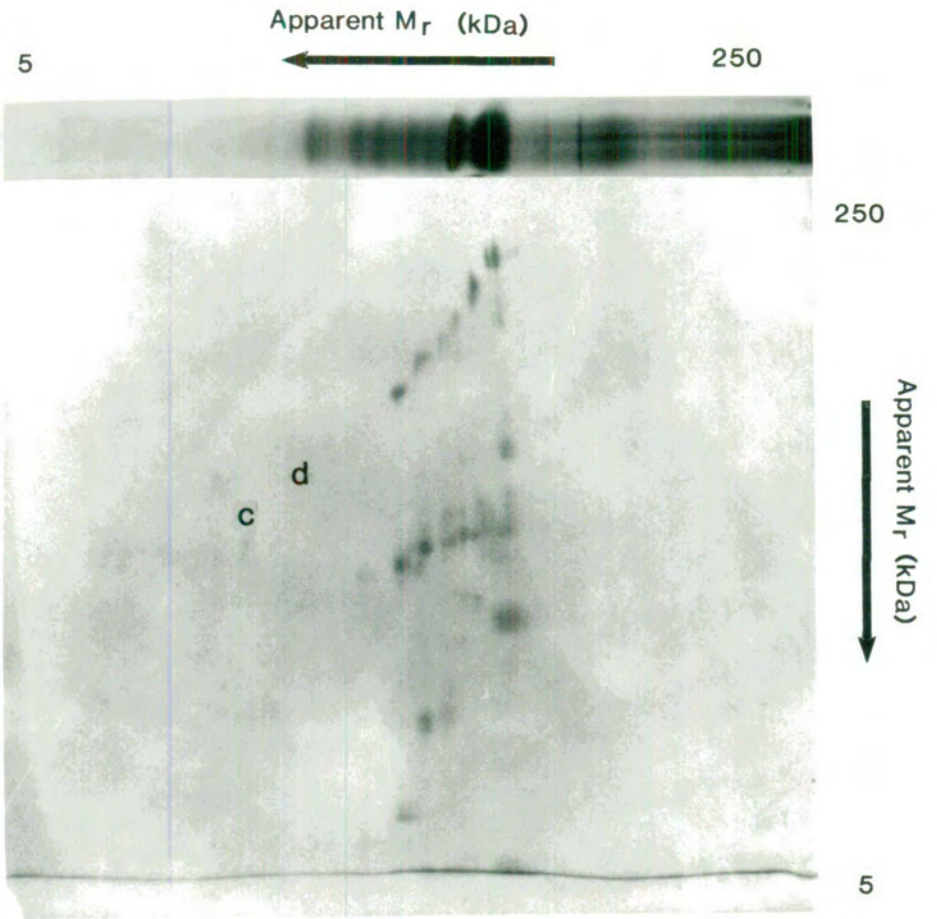

Fig. 8.2(a) Tryptic cleavage pattern of hair proteins from goat (Angora). Proteins were S-carboxymethylated with ^{14}C-IAA and the gel stained with Coomassie blue. High cysteine-rich protein bands are indicated by c and d.

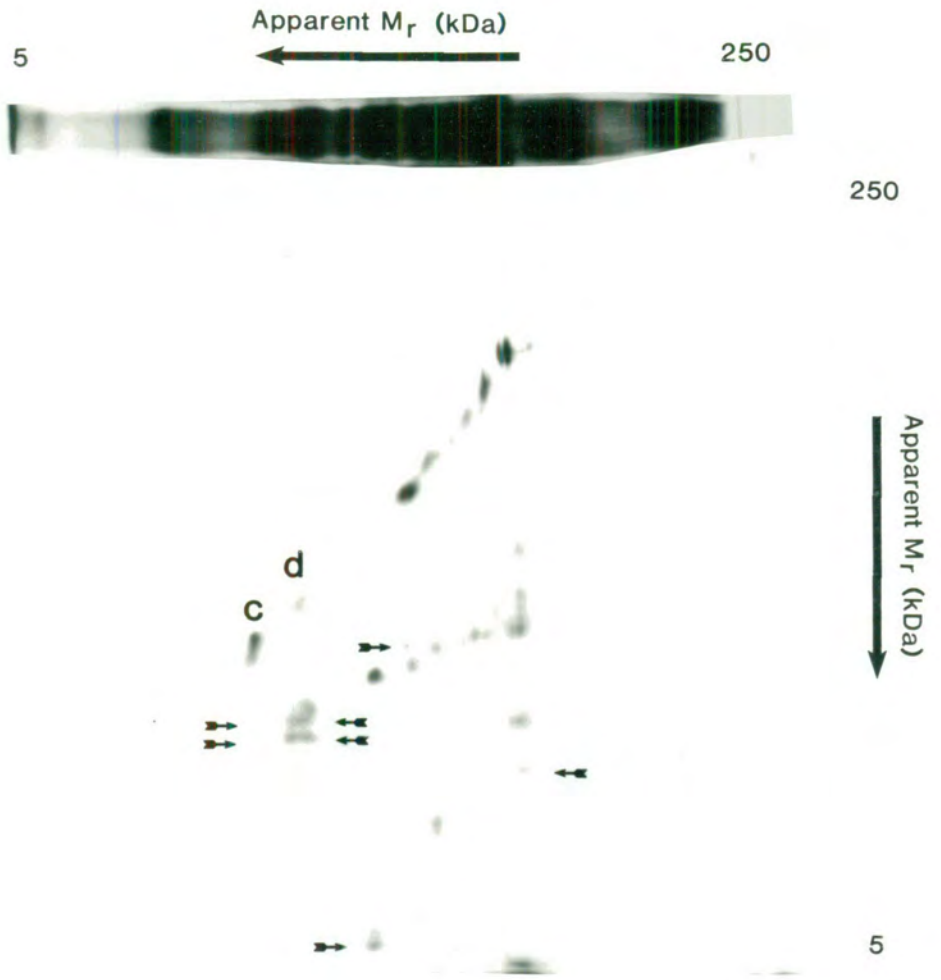

Fig. 8.2(b) Fluorograph of the same sample (goat) as represented in Fig. 8.2(a). High cysteine-rich protein bands are indicated by c and d. Arrows indicate differences between Coomassie blue stained gel and fluorograph.

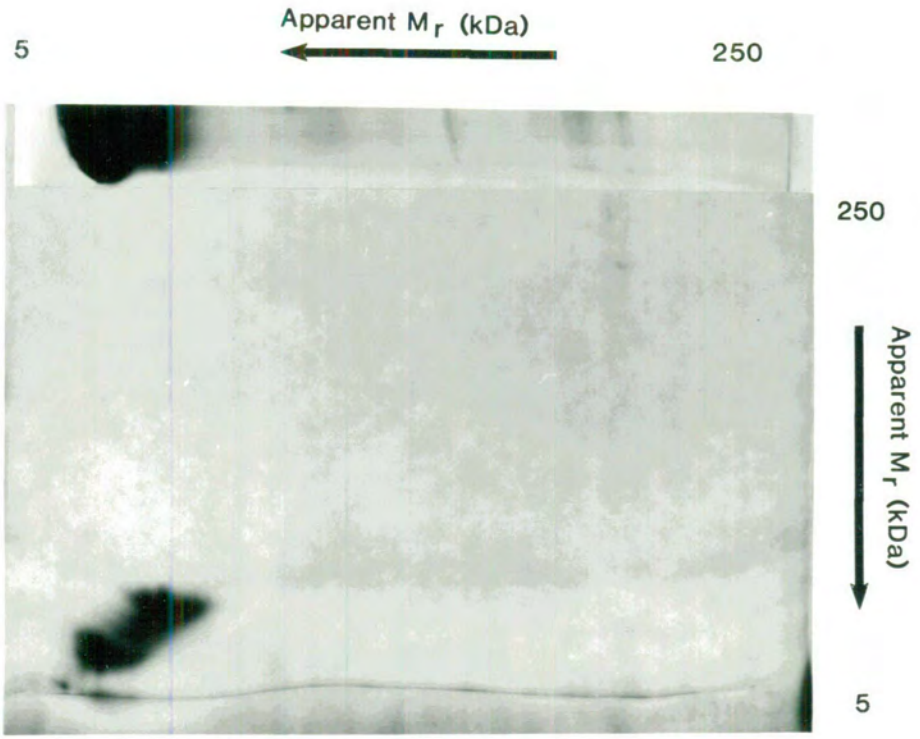

Fig. 8.3(a) Tryptic cleavage pattern of feather proteins from chicken. Proteins were S-carboxymethylated with ^{14}C-IAA and the gel stained with Coomassie blue.

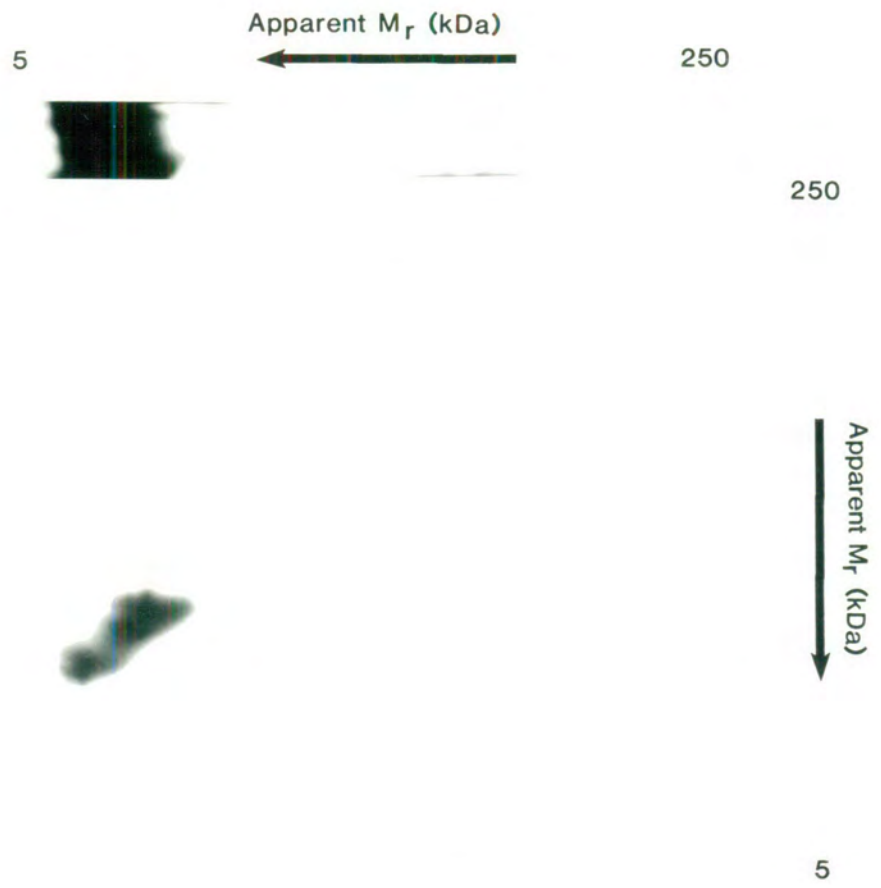

Fig. 8.3(b) Fluorograph of the same sample (chicken) as represented in Fig. 8.3(a).

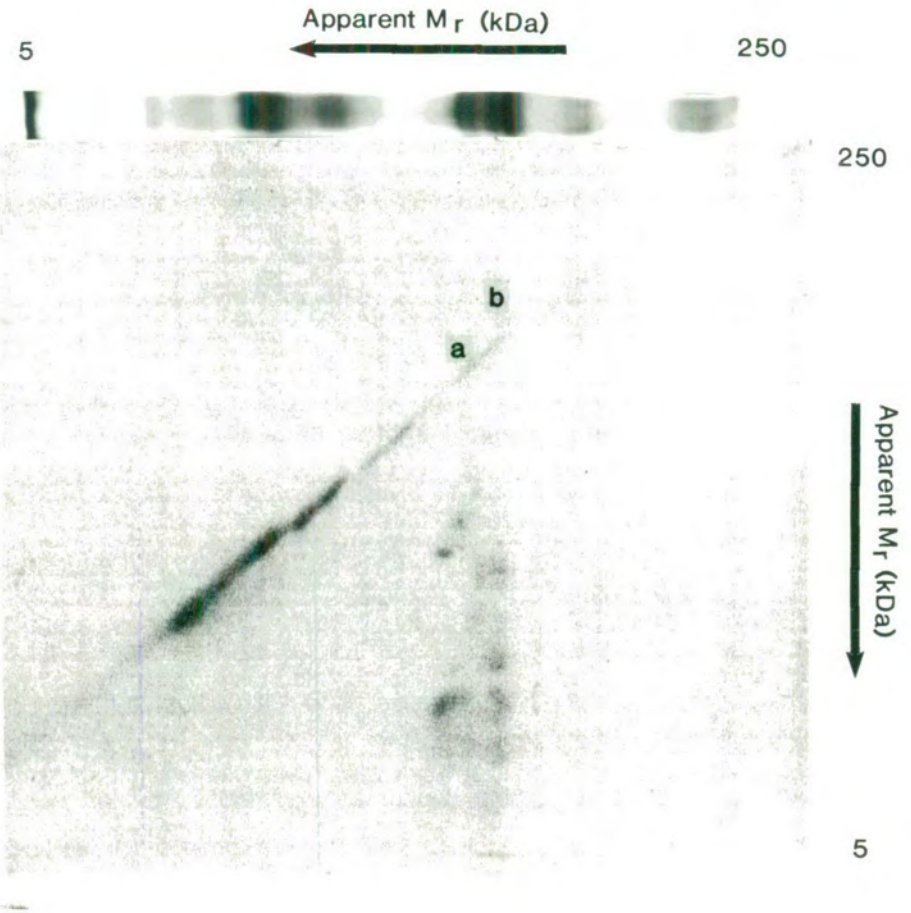

Fig. 8.4(a) Fluorograph of hair proteins from rat. Mid-range molecular mass protein bands are indicated by a and b.

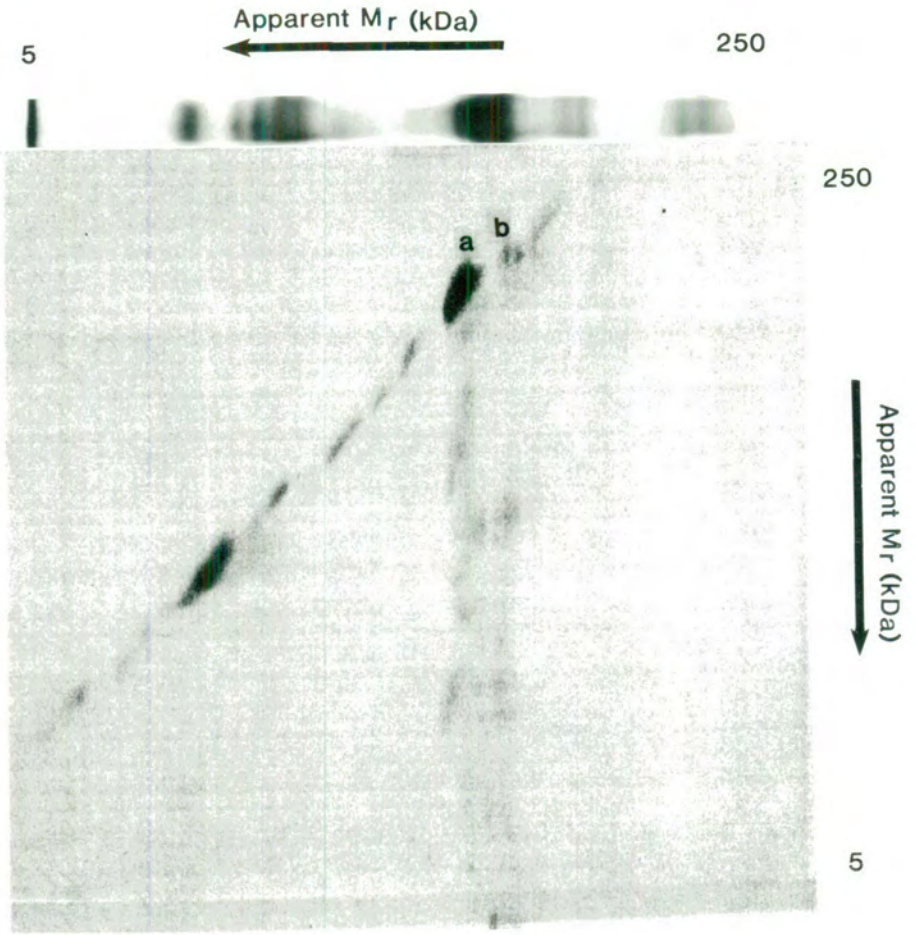

Fig. 8.4(b) Fluorograph of hair proteins from possum. Mid-range molecular mass protein bands are indicated by a and b.

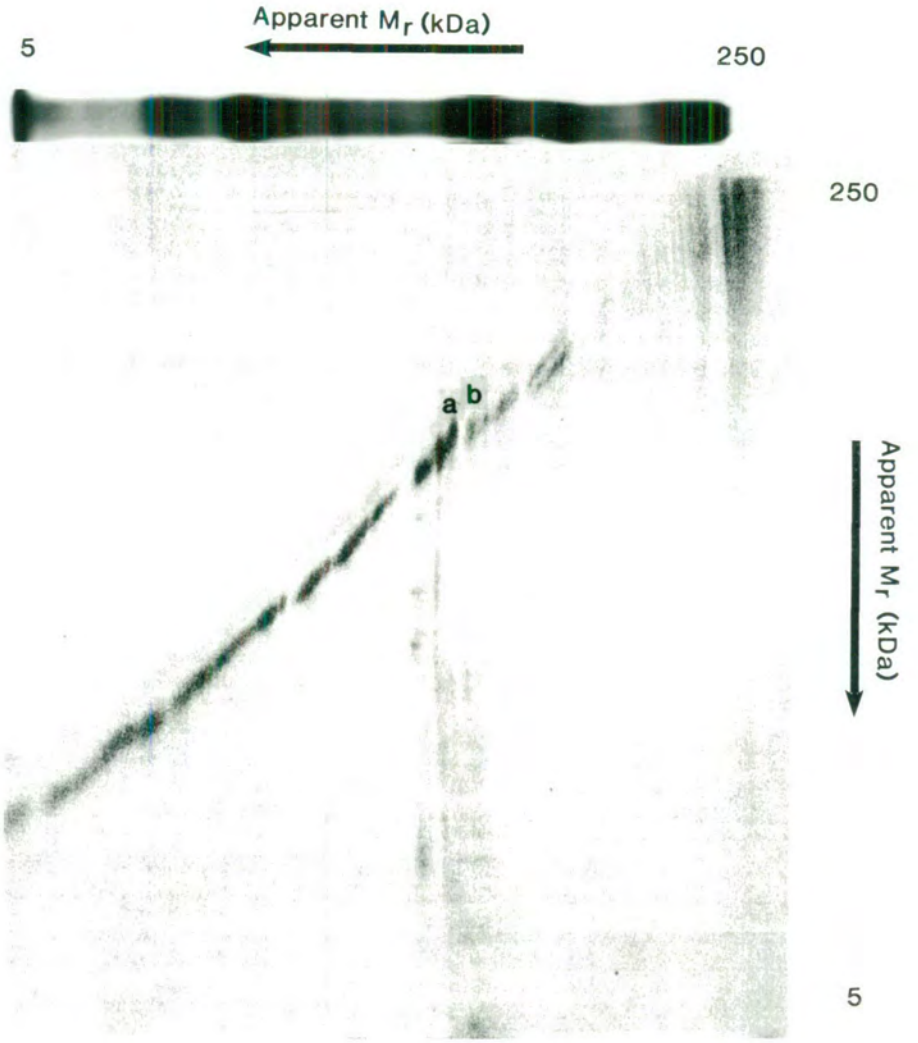

Fig. 8.4(c) Fluorograph of hair proteins from mountain pygmy possum. Mid-range molecular mass protein bands are indicated by a and b.

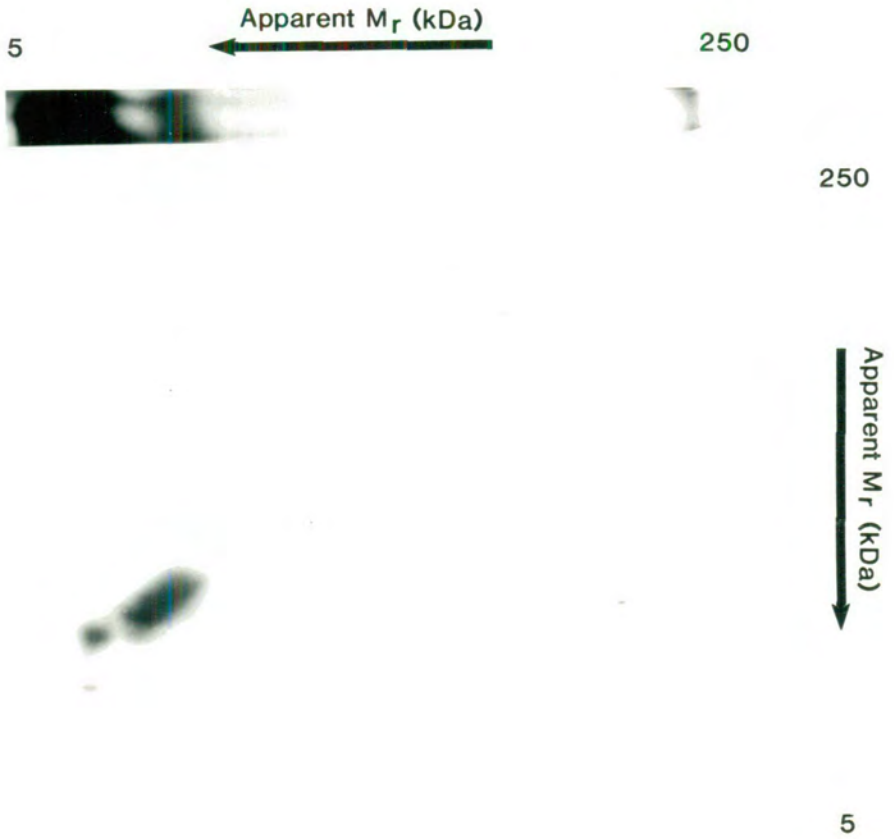

Fig. 8.4(d) Fluorograph of feather proteins from duck.

8.3 DISCUSSION

Sodium dodecyl sulphate gel electrophoresis coupled with partial proteolysis is a useful tool for the characterisation, cleavage and/or isolation of polypeptides from proteins of different species of human, animals and birds (Cleveland *et al.*, 1977). The limitation of the Cleveland procedure is that only single discrete protein bands are digested to generate the peptide pattern. Bordier and Crettol-Jarvinen (1979) described a method where reduced and denatured peptides derived from the proteins of a complex mixture separated in the first dimension by SDS gel electrophoresis after equilibration in stacking gel buffer and subsequent proteolysis were separated in the second dimension. In terms of economy, ease of manipulation and reproducibility the generation of a peptide profile from a complex but recognisable mixture of proteins (such as the S-carboxymethylated kerateines of hair) offers several experimental and descriptive advantages. The method used in the present study, involves the partial digestion of radioactive S-carboxymethylated proteins which have been separated in the first dimension by SDS gel electrophoresis. The peptide pattern is also characteristic of each species although it is possible to identify differences within species. We have shown that the technique may be used with gel slices which have been previously fluorographed and stored dry for up to six months. Not only is this very convenient in dealing with large number of samples, it also provides for considerable savings in terms of radiolabel, experimental manipulation and electrophoretic reagents.

Peptide patterns of two human samples (Australian European of Anglo-Celtic origin and Australian Aboriginal) are shown in Fig. 8.1(a). The separation of the undigested proteins along the diagonal is much clearer than the separation normally seen with a standard 1D gel (Khawar *et al.*, 1995). This provides a clear demonstration of the molecular mass heterogeneity of substituted keratins from human hair. As it is clear from Fig. 8.1a, b, c, cysteine-rich domains have been produced by trypsin cleavage in both the high and mid-molecular mass region (labelled a, b in Fig. 8.1a, b, c) in the case of humans. Human samples (Fig. 8.1a, b and c) showed a number of high molecular mass, high cysteine proteins cleavable by tryptic digestion which were not seen in goat and other mammals.

The very high cysteine proteins of goat hair (labelled c, d in Figures 8.2a and 8.2b) in the low molecular mass region did not appear to be quite as resistant to trypsin digestion as the human proteins of similar molecular mass. The tryptic pattern of human and goat hair proteins is however similar in the sense that both demonstrated many cleavage fragments in the mid-molecular mass range (labelled a, b). The two human samples from Australian European and Australian Aboriginal showed small differences in the peptide fragments in the mid-molecular mass region as indicated by the arrows in Fig. 8.1b. The peptide pattern of humans is quite different from other

mammals like rat (Fig. 8.4a), possum (Fig. 8.4b) and mountain pygmy possum (Fig. 8.4c). In humans there is a very close relatedness of tryptic digestion bands in the higher molecular mass high cysteine proteins indicated and bracketed in Fig. 8.1a and 1b suggesting that the entire cluster represents variously truncated variants of a single gene product. Mid range bands (a, b) are also closely related since they give many of the same peptide fragments. Other lower molecular mass high cysteine bands are probably all separate gene products since the tryptic cleavage fragments are all different. A similar cluster of relatedness particularly in the high molecular mass area was also observed in peptide mapping of baboon and chimpanzee hair proteins (not shown here). These polydisperse high molecular mass, high cysteine proteins, apparently derived from a single gene product are not seen in other mammals we have studied and may therefore be of relatively recent evolutionary origin.

Fig. 8.2a and 2b show the tryptic fragmentation pattern of goat, Coomassie blue stained gel and autoradiograph respectively. The pattern showed relatedness between low molecular mass (c, d) proteins (some but not all identical fragment products) but other proteins showed dissimilar patterns. The very high molecular mass, high cysteine proteins so prominent in humans and other primates were not apparent in the goat profile or indeed the profile from other mammals studied.

Tryptic fragmentation products for rat, possum and mountain pygmy possum are shown in Fig. 8.4a, b, c respectively. Whereas the mid-range molecular mass proteins generated a variety of tryptic fragments many of the low molecular mass proteins, like proteins of similar molecular mass in humans, and other mammals studied, while particularly rich in cysteine seemed to be almost as resistant to trypsin digestion as the even lower molecular mass keratins in the feathers from chicken and duck (Fig. 8.3a, b; Fig. 8.4d). Clearly, although the patterns shown by the possum and the mountain pygmy possum were quite similar (particularly in regard to the homologous fragmentation products derived from proteins a & b in Fig. 8.4(b) and (c) respectively) they nonetheless also demonstrate differences much more substantial than those shown between the two human samples (Fig. 8.1a, b, c).

The patterns for feather keratins observed in the case of chicken and duck (Fig. 8.3a and 3b; Fig. 8.4d) were quite dissimilar to any of the mammalian hair patterns. Only 7-8 protein spots were seen in the very low molecular mass region for the chicken and only 3-4 spots for duck in the same region. A study made by O'Donnell and Inglis (1974) found that feather keratins of two distantly related birds like emu and silver gull had similar sequence giving rise to the notion that feather keratins are more tightly conserved than mammalian keratins. Our preliminary results also confirm this notion showing the relatively simple subunit structure of the feather proteins almost

proteins almost exclusively in the low molecular. mass region (around 10 kDa), a point previously remarked upon by other authors (Brush, 1974; Knox, 1980).

Overall these 2D separations show some improvements on previously reported proteolytic digests (Marshall, 1980). Clearly much scope remains for the development of protease cleavage fragment signatures of hair and feather proteins using other proteases (*S. aureus* V8; α-chymotrypsin for example) and this is being actively pursed this in our laboratory.

GENERAL DISCUSSION

Hair is a better indicator of total body burden of some metals than blood or urine (Hammer *et al.*, 1971). Hair has some advantages as it is supposed to provide a better reflection of some metals like Hg as levels of Hg in hair are more stable than in other tissues (Phelps *et al.*, 1980). Analysis of human hair is thus an experimental technique that has clinical and forensic promise. There may be a possible relationship between the nature and disposition of hair proteins and the rate of heavy metal accumulation. The hair proteins mainly reside in histological components that are the structural parts of hair (Gillespie, 1983). To make these proteins soluble for further analysis, very harsh conditions are required as the cleavage of disulphide bonds (largely present between cysteine residues of hair) resist the breakdown. Hair proteins can be extracted by reducing agents and chemically converted to their more stable derivatives S-carboxymethyl kerateines (SCMKs). Factors like nutritional deficiencies (Gillespie, 1966), chemical treatments (Marshall, 1983) and genetic disorders (Gillespie and Marshall, 1983) may contribute toward the modification of SCMK proteins. This, in turn, may be responsible for abnormalities in the structure of hair (Nappe and Kermici, 1989). Such epigenetic variation should be considered alongside true genetic polymorphism which may alter the protein milieu from person to person and in the wider context from one racial group to another (Miyake and Seta, 1990).

In the first part of the heavy metal analysis of the present study, the mean concentrations of each of the 14 elements (Mg, Al, Cr, Fe, Mn, Co, Ni, Cu, Zn, As, Se, Cd, Hg and Pb) were compared between each individual test village in the vicinity of the gold and copper mine at Ok Tedi and the global mean of the control villages (individuals distant from the mine site but still of the same racial type (Melanesians)). Although there is an increase in the levels of metals like Cr, Zn, Ni, Co, Cd, Hg and Pb in the Ok Tedi villages with respect to the control site (Central Highlands), these slightly elevated levels are still well within the previously published "normal" values for rural and urban "Western" populations. The increase in the levels of Zn in hair of the Ok Tedi population probably reflects the improvement in nutrition as the population moved from a marginal hunter gatherer existence to almost total reliance on processed food supplied by the mining consortium. This conclusion is supported by the concommitant sharp rise in the levels of Hg in the Ok Tedi population since the major source of food protein is now tinned fish provided by the mining company. The general increase in Cu levels at Ok Tedi is to be expected but even after several years of intensive copper production is certainly well within previously published levels for other (Western) populations in non-mining areas (Reilly and Harrison, 1979).

In the second part of the heavy metal analysis, 14 elements were compared between and among four different groupings (controls, Ok Tedi tests, Australian Aboriginals and Australian Europeans). Overall, the results of this study show that increases in the levels of some elements like Cr, As, Se, Cd, Hg and Pb at the test sites may be the result of mining activity as well as the changing trend in lifestyle and diet. A slight increase in some of the toxic elements including Cd, As, Hg and Pb is seen in the test sites by comparison with the control (Melanesian) population. These "elevated" levels are, however, in close agreement with the levels shown by Australian Europeans living in a presumptively non-polluted environment (Armidale, NSW) which in turn are in close agreement with previously published values for other Westernised populations.

On the other hand, the Australian Aboriginal group (living close to a manganese mine in Groote Eylandt) clearly show average hair Cu, Se, Cd, Hg and Pb levelss which are of concern, and may suggest environmental pollution, some peculiarity of the hair protein or some drastic lifestyle change in this particular population. For example, the results of hair Hg shown by Australian Aboriginals are approaching results shown by a population of gold miners in Brazil (Malm *et al.*, 1990). These values for Australian Aboriginals are nevertheless still lower than the results (up to 40.0 and 31.8 mg/g) shown by both Indians and gold miners respectively, supporting the notion that the actual load strongly depends on the dietary and other habits of each individual (Malm, *et al.*, 1990). It should also be mentioned here that the hair Hg accumulation, in particular, may depend to an extent on the nature and disposition of hair keratins.

In the third and final part of the hair heavy metal analysis the longitudinal changes that have occurred during a period of six years (1984-1990) of heavy mining operation at the Ok Tedi (test) sites have been discussed. This survey includes the monitoring of three essential metals Fe, Cu and Zn and three non-essential metals Pb, Cd and Hg.

Given the correlations we have previously observed, it is reasonable to conclude that mining activities *per se* bear, at most, only second-order responsibility for the marked changes in hair heavy metals from 1982 (premining) to 1990. Changing diets and lifestyle patterns may very well result in an increase in some essential metals like Cu and Zn and non-essential metals like Pb and Hg. These values (Ok Tedi 1990) are nevertheless well within normal ranges previously observed for Western populations in non-mining areas (Reilly and Harrison, 1979; 1983). It appears that with regard to the previously published values, the increases seen in the Ok Tedi population from 1982 to 1990 reflect a shift from a profile of the typical Melanesian control group (Mt. Obree, 1990) to a profile typical of a rural Westernised population (Armidale, 1993).

As previously mentioned, there may be a possible relationship between hair protein and heavy metal accumulation. SDS-PAGE offers a most convenient technique for the analysis of human and animal hair and feather proteins. The present study shows that a reproducible electrophoretic pattern can be obtained by 1D-SDS-PAGE. More than 400 human hair samples were analysed by 1D-SDS-PAGE and at least 20 components could be separated electrophoretically according to their molecluar masses (see Chapter 6). Furthermore, the relative cysteine content of each zone was estimated by comparing the laser densitometer traces for Coomassie stained gels with corresponding autoradiographs. This proved of considerable comparative value despite the caveat of variations in the uptake of Coomassie dye by S-carboxymethylkerateines (Gillespie, 1991). Overall, 1D 12.5% SDS-PAGE separation of SCMKs of human hair proteins represents an improvement on previously published 1D separations of human hair proteins. This technique can be used for the mass screening of hair samples belonging to different racial groups using Coomassie stain, silver stain and fluorography. We have used this technique to show that Australian Aboriginals have a characteristic distribution of a high cysteine protein electromorph and this is, in part, may account for the unusually high levels of Hg and other metals seen in hair from this group (Chapter 3). In addition, this procedure was also applied to the analysis of animal hair and feather keratins. The suitability of this 1D technique was demonstrated for the simultaneous multiple comparison of mammalian and avian feather proteins with respect to apparent molecular mass and relative cysteine content of their S-carboxymethylated proteins. A note of caution should be sounded here, however, with regard to interpretation of protein profiles. It is conceivable that at least some of the differences we have observed in protein profiles, both within and between species, are the result of differential solubilisation of key proteins.

Allowing for this caveat and noting the uniformity of extraction procedures the SDS-PAGE profiles (see Chapter 7) indicate that each animal showed a characteristic and reproducible protein pattern. All primate (human, gorilla and baboon) patterns seemed to very similar to each other and goat and sheep were also closely related. The placental mammal pattern as a group again showed closer internal resemblance as did the marsupial mammals. On the other hand, protein patterns shown by birds (chicken and duck) are completely different from all mammals (placentals and marsupials). This supports the notion that bird feathers are derived through a completely different lineage to mammalian hair (Brush, 1974). We believe that improved 1D separation and analysis used in this survey of human, animal hair and feather proteins may be helpful in both a taxonomic and forensic context (Hrdy and

Baden, 1973; Marshall, 1985), as well as casting light on the mechanisms by which heavy metals are accumulated in the hair.

The final section of this thesis deals with the peptide mapping of S-carboxymethylated proteins of human and animal hair and feather proteins by 2D gel electrophoresis. Again, human hair proteins from different races after proteolytic digestion show more or less similar tryptic cleavage patterns with minor differences. Although goat hair proteins show some differences from human protein profile, these patterns are similar in the sense that many cleavage fragments can only be seen in the mid-molecular mass range (see Chapter 8). Very high molecular mass high cysteine proteins which are clearly present in the human profile were not seen in the goat profile or the profile from all other mammals studied with the exception of other primates. The patterns for feather keratins observed from chicken and feather proteins were completely different from mammalian hair patterns in that a few protein spots can be seen only in the very low molecular mass region. These preliminary results confirm the previous notion of a simple subunit structure of feather keratins (Brush, 1974; Knox, 1980).

In short, these 2D separations represent an improvement on other previously published proteolytic digests (Bordier and Crettol-Jarvinen, 1979; Marshall, 1980) but there is some scope of using other proteases (*S. aureus*; α-chymotrypsin) which would be the subject of further investigations in the application of hair protein analysis to ontogenetic and forensic studies.

CONCLUSIONS AND INDICATIONS FOR FUTURE WORK

1) It is concluded from the trace element study of human hair of populations from the Ok Tedi (test sites) and Central Highlands (control sites) that although there is a consistent elevation in the levels of several metals (Cr, Zn, Ni, Co, Cd, Hg and Pb) with respect to the Melanesian control group, these elevated levels are still within previously published "normal" ranges. The increase in hair Zn in the Ok Tedi population should be seen as a positive change reflecting improved nutrition.

2) Overall results of trace elements in the Ok Tedi (test sites), Central Highlands (control sites) as well as Australian Aboriginals and Australian Europeans showed that increase in the levels of some elements (Cr, As, Se, Cd, Hg and Pb) at the test sites should be seen both in the context of mining activity **and** the rapidly changing trend in lifestyle and diet. The mean levels of some toxic elements like Cd, As, Hg and Pb did rise in the Melanesian (Ok Tedi) test population but rarely exceeded the levels seen in Australian Europeans living in a non-industrial rural city (Armidale). On the other hand, the mean levels of Cu, Se, Cd, Hg and Pb in the Australian Aboriginal group were so clearly elevated above all other groups as to provide grounds for health related concerns

3) It appears reasonable to conclude that mining activities *per se* was less responsible for the marked changes in hair heavy metals from 1982 (premining) to 1990 in the Ok Tedi population, then changing lifestyles that may result in an increase in some of the heavy metals. The shift in the Ok Tedi group from a pattern typical of a rural Melanesian population (Central Highland, 1990) in a non mining setting to a pattern more typical of a rural Westernised population (Armidale, 1993) is not of any particular health concern in the light of previously published reports.

4) The 1D-SDS-PAGE separation of S-carboxymethylated human hair proteins represented here provides information about the variety of human hair proteins and, in conjunction with laser densitometry, can be used to demonstrate phenotypic variation in various human populations. The possible differential solubility of key proteins from the hair of individuals, particularly from different racial groups, could lead to some difficulties in interpretation of possible phenotypes. Future work will resolve this question by quantitating the solubility of different hair types in families whose genetic relationships and hair protein profile are known.

5) Finally, the 2D-separations of proteolytic digests of separated whole keratin mixtures reported in this study show considerable promise in the establishment of a hair peptide fingerprint.

Future work may involve investigation of possible correlations between protein polymorphism and heavy metal levels in different populations. Sequencing of cysteine-rich domains of human and animal hair and avian feather proteins after proteolysis, 2D SDS-PAGE and Western blotting is likely to yield informative phylogenetic results. There

is also a need to expand such studies to include sequencing of reptile and fish scale proteins to establis evolutionary relationships. Furthermore, population genetic and family analysis of hair protein polymorphisms including all major races with appropriately large sample number including peptide mapping may be useful in a forensic context. Much scope still remains for the development of protease cleavage fragment signatures of hair and feather proteins using other proteases (*S. aureus* V_8; α-chymotrypsin; pronase etc.) which may prove of value in evolutionary, ontogenetic and forensic science.