Loss of a Homologous Group of Proteins in a Dominantly Inherited Ectodermal Malformation

By HARRIET S. TENENHOUSE and REYNOLD J. M. GOLD

The deBelle Laboratory for Biochemical Genetics, McGill University, Montreal Children's Hospital Research Institute, 2300 Tupper Street, Montreal, Quebec H3H 1P3, Canada

(Received 8 April 1976)

Hair from mice bearing the dominantly inherited Naked trait (*Nn*) and from normal (*nn*) mice of the same inbred strain was separated into its major protein components by standard techniques. The relative amounts of proteins in these components were then determined by a regression method from the amino acid composition of the hair samples and of the fractions into which they had been separated. The results indicated that the amount of soluble fibril in Naked-mouse hair is decreased. Polyacrylamide-gel electrophoresis of this fraction prepared from the hair of both normal and Naked mice revealed that all protein bands present in the normal are also present in the Naked mice. However, a densitometric scan of the gels at 280 nm showed that the soluble fibril fraction from Naked-mouse hair is deficient in several proteins which, on amino acid analysis, were found to contain 31% glycine and 10% tyrosine. Gel filtration of *S*-carboxymethylkerateine prepared from normal and mutant hair showed that the mutant hair is deficient in a heterogeneous, low-molecular-weight fraction also rich in glycine and tyrosine. Our present data do not reveal the mechanism whereby a single gene locus modulates the production of several different proteins.

Although many dominantly inherited malformations of man have been described (McKusick, 1971), few have been characterized at a molecular level since, in most cases, the phenotype provides no intelligible clues to the underlying molecular defect. In the dominantly inherited ectodermal dysplasias, however, keratin suggests itself as the protein primarily affected by the mutation, and indeed chemical abnormalities in human ectodermal dysplastic keratin have been reported (Scriver *et al.*, 1965; Gold & Scriver, 1971, 1972; Gold & Kachra, 1974). Here we report studies of an ectodermal malformation segregating in an inbred strain of mice so that any biochemical abnormalities found can be attributed with confidence to the mutant gene.

The Naked mutation of mice has been previously described (Lebedinsky & Dauvart, 1927; David, 1932; Steinberg & Fraser, 1946). Mice heterozygous at the Naked locus (*Nn*) show patchy depilation caused by breakage of the hair close to the root. Mutant homozygotes (*NN*) die *in utero* or shortly after birth. Tenenhouse *et al.* (1974) have shown that hair from *Nn* mice contains 20% less glycine and 30% less tyrosine than hair from normal (*nn*) mice of the same inbred strain. This finding strongly suggested that Naked-mouse hair is deficient in the high-glycine, high-tyrosine (HG) class of proteins present in all keratins and particularly abundant in mouse hair (Gillespie, 1972). DeDeurwaerder *et al.*

(1964) were the first to extract HG proteins preferentially from wool, and electron-microscopic studies suggested that these proteins are located in the intercellular membrane complex. However, Fraser *et al.* (1973) have shown that the HG proteins also occur in the matrix region of keratin fibres and have suggested that they play a specific structural role in all hard keratins.

The experiments reported here suggest that Naked-mouse hair has lost a large proportion of its soluble fibrillar component and that the protein lost is largely of the HG type.

Materials and Methods

Mice

The mice used were of the NS-FR strain inbred by brother-sister mating for 57 generations. Only heterozygotes for the Naked gene were mated.

Chemicals

2-Mercaptoethanol, iodoacetic acid, 2-amino-2-methylpropane-1,3-diol, acrylamide, *NN'*-methylene-bisacrylamide and *NNN'N'*-tetramethylethylenediamine were from Eastman Organic Chemicals Distillation Industries, Rochester, NY, U.S.A. Sephadex G-75, bovine ribonuclease and chymotrypsinogen were from Pharmacia, Uppsala, Sweden.

AG50W-X8 (200–400 mesh) was from Bio-Rad Laboratories, Richmond, CA, U.S.A. All common chemicals were from Fisher Scientific, Montreal, Canada.

Preparation of hair

Samples of hair shaft were taken from the mice, washed with water, defatted with light petroleum (boiling range 38.1–47.1°C) and air dried.

Hydrolysis of proteins and amino acid analysis

Hair samples and protein fractions derived from hair were hydrolysed in 6M-HCl for 24 h at 110°C and analysed on a Jeol amino acid analyser as described previously (Tenenhouse *et al.*, 1974).

Fractionation of hair proteins

The fractionation of mouse hair is summarized in Scheme 1. Washed mouse hair (*a* in Scheme 1) was suspended in the reduction buffer, 6M-urea/0.2M-mercaptoethanol/0.2M-2-amino-2-methylpropane-1,3-diol, pH 10.5 (12 ml/50 mg of hair), and incubated under N₂ for 3.5 h at 50°C with shaking. The mixture was then centrifuged and the residue (*b*) was washed with water to remove all soluble proteins, and air dried. The supernatant was treated with 0.55M-iodoacetic acid (1 ml/50 mg of hair), which was added dropwise with stirring. During alkylation, the pH was maintained at 9 with 5M-NaOH. Excess of iodoacetic acid was removed by addition of 2-mercaptoethanol until free thiol could be detected (positive nitroprusside test). The *S*-carboxymethylkerateine thus prepared (*c*) was dialysed for 24 h against 10 vol. of water, which was then kept for further analysis as described below. All dialyses were for a minimum of 16 h. The *S*-carboxymethylkerateine was twice dialysed against 6 litres of water and a portion of it was freeze-dried. The remaining *S*-carboxymethylkerateine (*c*) was made 0.02M with respect to zinc acetate, stirred for 2 h and then centrifuged for 15 min at 3000g. The zinc acetate supernatant (*d*, matrix) was dialysed twice against 6 litres of water and freeze-dried. The zinc acetate precipitate (*e*, fibril) was freed from soluble proteins by washing several times with 0.02M-zinc acetate, dissolved in 0.02M-sodium citrate and finally dialysed against water. The fibril fraction (*e*) thus prepared was then partitioned into the insoluble fibril (*f*) and soluble fibril (*g*) by the methods described by Gillespie (1972). All protein fractions (*a*–*g*) were hydrolysed in quadruplicate and the hydrolysates analysed for amino acid composition.

Analysis of the diffusible material from *S*-carboxymethylkerateine

To determine whether any losses occurred during the dialysis of *S*-carboxymethylkerateine, the solution

outside the dialysis bag was analysed as follows. After freeze-drying, the product was dissolved in 5 ml of water and the pH was adjusted to 7 with NaOH. To remove the buffer components from any amino acids and peptides, the product was applied to an AG-50 column (H⁺ form). The components of the reduction buffer were eluted by extensive washing of the column with water. Any peptides or amino acids present in the product, were then eluted with a 2M-NH₃ wash, which, in control experiments, was found to elute amino acids and dipeptides completely. Under these same conditions, an 83% recovery of a model hexapeptide was obtained. The fraction eluted with 2M-NH₃ was evaporated and washed three times with water to remove the NH₃ and hydrolysed for amino acid analysis. Control experiments were performed to determine base-line values arising from residual buffer components or from the AG-50 resin (Greenland *et al.*, 1974).

Stability of proteins during extraction

It was necessary to determine whether the heterogeneity of the HG proteins extracted from hair was a result of the extraction procedures used. The reduction was therefore carried out for 40 min and

Scheme 1. Scheme of hair fractionation

6h, in addition to the 3.5h described above, before alkylation with iodoacetic acid. The proteins extracted (c) under these conditions were subjected to gel filtration on Sephadex G-75 as described below. Appropriate fractions were pooled for amino acid analysis and polyacrylamide-gel electrophoresis.

Polyacrylamide-gel electrophoresis

Electrophoresis was carried out at pH 8.9 in 6M-urea with a 7% running gel, by the method of Davis (1964). Equal weights of protein were applied to all gels. After electrophoresis, the gels were washed with 10% (w/v) trichloroacetic acid to remove urea and precipitate the protein bands. A densitometric scan of the gels was performed at 280nm with a Gilford spectrophotometer. To extract the proteins, the gels were cut into sections and placed in 1M-NH₃ and homogenized by hand with a Teflon-pestle homogenizer. The gel was removed by centrifugation at 8000g for 10 min and the proteins thus extracted were dialysed twice against 1000 vol. of water and freeze-dried before hydrolysis.

Gel filtration

S-carboxymethylkerateine (c) (30.8 mg, 26.1 E_{280} units), extracted from normal mouse hair, was dissolved in 1.5 ml of 0.1M-sodium phosphate/6M-urea, pH 7. The solution was applied to a Sephadex G-75 column (90 cm $\times$ 2 cm) equilibrated in the same buffer. Fractions (4 ml) were collected, and monitored at 280 nm. Appropriate fractions were pooled, dialysed twice extensively against 6 litres of water and freeze-dried for further investigations. A similar solution containing 30.0 mg (22.8 E_{280} units) of S-carboxymethylkerateine (c) prepared from Naked-mouse hair was eluted under the same conditions.

Results

Amino acid composition of keratin fractions from normal and Naked-mouse hair

The amino acid composition of normal and Naked-mouse hair and of the fractions derived from them are shown in Table 1. As reported previously, Naked-mouse hair contains 20% less glycine and 30% less tyrosine than hair from normal mice of the same inbred strain (Tenenhouse *et al.*, 1974). Table 1 shows that these differences persist in all the extracted fractions [except that there is no significant deficiency of glycine in fraction (f) of Naked-mouse hair]. There is no significant difference in the glycine and tyrosine content of the insoluble residues (b). In addition, there are other significant differences in the extracted fractions. For example, the fibril fraction (e) prepared from Naked-mouse hair contains

significantly more threonine, glutamic acid, proline and valine and significantly less phenylalanine than do normal fibrils. All these changes are consistent with the hypothesis that the fibril fraction from Naked-mouse hair is deficient in HG proteins, since these proteins are known to contain less threonine, glutamic acid, proline and valine, and more glycine, tyrosine and phenylalanine than whole hair (Gillespie, 1972). The similar amounts of glycine and tyrosine present in the residues (b) obtained from normal and Naked-mouse hair are also consistent with our expectation, since the HG proteins are known to be extractable by the procedures used (Gillespie, 1972) and hence would not be present in the residue. However, there are significant differences in the residue. For example, the residue prepared from mutant hair contains abnormally low amounts of glutamic acid. This suggests that minor changes in protein composition, other than the major loss of HG proteins, have occurred.

Relative amounts of major components in normal and mutant keratin

The amino acid-composition data shown in Table 1 were subjected to regression analysis as described in the Appendix (Gold *et al.*, 1976) and generated the results shown in Table 2. Table 2 shows that Naked-mouse hair contains only 69.7% extractable protein, compared with 78.6% for normal hair, and that this deficit is largely accounted for by a decrease in the amount of soluble fibril.

The regression method [see the Appendix (Gold *et al.*, 1976)] requires that all amino acid residues originally present in the mixture enter either one or the other fraction into which it is separated and are not, for example, lost during dialysis. We found that losses of amino acid residues during dialysis of S-carboxymethylkerateine (c) prepared from either normal or Naked-mouse hair were no greater than 0.3% of the amino acid residues present in the original sample. Since no degradative procedures were used subsequent to the reductive extraction of hair, it was assumed that no further losses would occur in subsequent dialyses.

Loss of HG proteins from the soluble fibril fraction

Since the principal deficit was found to be in the soluble fibril fraction, these fractions prepared from normal and Naked-mouse hair were therefore compared by subjecting equal weights to polyacrylamide-gel electrophoresis. Scanning the gels at 280 nm (Fig. 1) reveals that all protein peaks present in normal soluble fibril are also represented in the corresponding fraction derived from Naked-mouse hair. However, there are several cases where the peak generated by Naked hair protein has a lower E_{280}

Table 1. Amino acid composition of whole hair (a) and fractions b-g for normal and Naked-mouse hair

The results are expressed as mol%, and represent, in each case, the mean of four determinations from four separate hydrolyses. The s.d. and significance levels of the differences are indicated: * $P < 0.001$; ** $0.001 < P < 0.005$; *** $0.005 < P < 0.01$. Values for †Cys or CmCys represent mol% of half-cysteine in the whole hair (a) (see Scheme 1) and residue (b) and mol% of S-carboxymethylcysteine in extracted proteins (c-g).

Amino acid	Composition (mol%)							
	Whole hair (a)		Residue (b)		Total extract (c)		Supernatant (matrix) (d)	
	Control	Naked	Control	Naked	Control	Naked	Control	Naked
Lys	3.55 ± 0.34	4.32 ± 0.48	7.77 ± 0.94	8.30 ± 0.81	1.81 ± 0.07	2.22 ± 0.37	0.59 ± 0.02	0.79 ± 0.49
His	1.13 ± 0.10	1.17 ± 0.09	1.59 ± 0.79	1.75 ± 0.38	0.69 ± 0.02	0.71 ± 0.14	0.66 ± 0.05	*0.49 ± 0.02
Arg	5.77 ± 0.49	5.53 ± 0.48	5.06 ± 0.42	4.39 ± 0.11	5.94 ± 0.24	5.92 ± 0.46	6.04 ± 0.22	5.99 ± 0.23
†Cys or CmCys	12.86 ± 0.61	13.93 ± 0.99	8.41 ± 1.61	8.14 ± 1.23	13.55 ± 0.55	15.68 ± 2.01	27.83 ± 1.56	29.57 ± 3.08
Asp	5.51 ± 0.18	5.56 ± 0.14	5.63 ± 0.37	6.30 ± 0.32	5.74 ± 0.15	5.76 ± 0.21	2.41 ± 0.05	2.16 ± 0.08
Thr	4.54 ± 0.12	4.75 ± 0.14	3.64 ± 0.11	*4.27 ± 0.12	5.09 ± 0.36	5.43 ± 0.31	7.90 ± 0.09	*7.40 ± 0.28
Ser	8.21 ± 0.53	8.61 ± 0.49	7.50 ± 0.29	7.90 ± 0.34	9.28 ± 0.17	9.67 ± 0.56	12.50 ± 0.12	*13.54 ± 0.63
Glu	12.82 ± 0.41	13.61 ± 0.51	21.11 ± 0.59	**19.36 ± 0.35	11.18 ± 1.97	11.53 ± 0.38	8.79 ± 0.25	8.99 ± 0.47
Pro	6.54 ± 0.20	*7.16 ± 0.27	5.11 ± 0.55	6.46 ± 0.48	6.85 ± 1.14	8.00 ± 1.02	12.28 ± 1.14	12.52 ± 0.77
Gly	11.75 ± 0.37	9.49 ± 0.27	8.99 ± 0.18	8.75 ± 0.27	12.76 ± 0.80	***9.79 ± 1.31	6.84 ± 0.28	**5.85 ± 0.18
Ala	4.55 ± 0.13	***4.44 ± 0.10	4.07 ± 0.19	4.47 ± 0.15	4.21 ± 0.39	4.47 ± 0.59	2.60 ± 0.07	**2.25 ± 0.08
Val	4.53 ± 0.12	4.75 ± 0.15	4.77 ± 0.65	4.57 ± 0.18	4.25 ± 0.20	4.40 ± 0.24	4.99 ± 0.45	4.70 ± 0.39
Met	1.10 ± 0.18	1.22 ± 0.28	1.39 ± 0.24	0.69 ± 0.48	1.97 ± 1.70	2.63 ± 1.42	0.42 ± 0.46	0.49 ± 0.34
Ile	2.63 ± 0.10	2.81 ± 0.09	2.47 ± 0.21	2.82 ± 0.15	2.72 ± 0.68	2.75 ± 0.80	1.87 ± 0.17	1.73 ± 0.25
Leu	6.49 ± 0.17	6.37 ± 0.16	7.34 ± 1.04	6.75 ± 0.09	5.52 ± 0.30	5.05 ± 0.13	2.01 ± 0.23	1.92 ± 0.21
Tyr	5.22 ± 0.37	*3.64 ± 0.22	2.09 ± 0.22	2.10 ± 0.33	6.07 ± 0.19	*4.05 ± 0.24	1.25 ± 0.06	**0.89 ± 0.13
Phe	2.75 ± 0.09	2.59 ± 0.08	3.08 ± 0.20	2.94 ± 0.22	2.37 ± 0.06	**1.92 ± 0.14	1.05 ± 0.06	**0.79 ± 0.08

Amino acid	Composition (mol%)			
	Precipitate (fibril) (e)		Soluble fibril (g)	
	Control	Naked	Control	Naked
Lys	2.94 ± 0.59	3.15 ± 0.05	2.96 ± 0.40	2.92 ± 0.04
His	0.92 ± 0.29	0.83 ± 0.03	0.72 ± 0.08	0.84 ± 0.07
Arg	6.18 ± 0.13	6.33 ± 0.34	6.12 ± 0.26	5.94 ± 0.45
†Cys or CmCys	8.12 ± 0.65	8.41 ± 0.37	7.96 ± 0.90	***11.53 ± 0.57
Asp	6.95 ± 0.39	7.60 ± 0.27	7.27 ± 0.23	7.30 ± 0.22
Thr	4.18 ± 0.10	*4.65 ± 0.04	4.47 ± 0.23	4.68 ± 0.21
Ser	7.85 ± 0.90	7.99 ± 0.24	8.03 ± 0.54	8.32 ± 0.73
Glu	11.78 ± 0.27	*13.35 ± 0.30	12.60 ± 0.41	13.09 ± 0.45
Pro	3.97 ± 0.32	***4.77 ± 0.13	4.93 ± 0.47	4.97 ± 0.34

Table 2. Relative amounts, in mol%, of the major components in normal and Naked-mouse hair

The percentages shown are derived from the data in Table 1 by methods described in the Appendix (Gold *et al.*, 1976).

Fraction	Percentage of total hair protein	
	Control hair	Naked hair
Residue	21.4	30.3
Matrix	23.0	24.8
Soluble fibril	49.0	37.4
Insoluble fibril	6.6	7.5

than the corresponding peak generated by normal hair proteins. The proteins corresponding to two of these peaks were subjected to amino acid analysis and found to have a high content of glycine and tyrosine, which is characteristic of the HG proteins. On the other hand, proteins that generate peaks of similar heights in the two genotypes contain far less of these two amino acids (see Fig. 1).

Molecular-weight profiles of extracted proteins

Since HG proteins are of low molecular weight (Gillespie, 1972), we looked at the molecular-weight profiles of *S*-carboxymethylkeratine (c) extracted from both normal and Naked-mouse hair. Fig. 2 shows the elution profiles obtained by applying *S*-carboxymethylkeratine from normal and Naked mice to a Sephadex G-75 column. Two major peaks are present. Peaks that correspond to material of high molecular weight are of similar intensities in the two samples. However, the peak corresponding to low-molecular-weight *S*-carboxymethylkeratines prepared from Naked-mouse hair have a lower *E*₂₈₀ than the corresponding peak derived from normal mouse hair. The glycine and tyrosine contents of the proteins in this peak, whether they originated from normal or Naked-mouse hair, are characteristic of the HG class of proteins. This, however, is not true of the higher-molecular-weight peak.

Heterogeneity of low-molecular-weight fraction

The heterogeneity of the HG proteins was evident when the low-molecular-weight fraction recovered from Sephadex G-75 (regions 3 and 4, Fig. 2) was subjected to polyacrylamide-gel electrophoresis. At least 12 protein peaks were resolved on the gels. The same 12 peaks were also observed with the low-molecular-weight fraction originating from Naked-mouse hair. To determine whether this heterogeneity was an artifact of reductive extraction, hair was extracted for 40min and for 6h in addition to the standard 3.5h. The low-molecular-weight fraction

Gly	14.64 ± 0.12	*11.68 ± 0.12	24.79 ± 2.28	20.80 ± 1.10	13.39 ± 0.09	*10.43 ± 0.71
Ala	5.15 ± 0.13	5.54 ± 0.22	3.57 ± 0.26	**4.55 ± 0.35	5.47 ± 0.22	5.74 ± 0.13
Val	4.47 ± 0.10	***4.89 ± 0.13	3.12 ± 0.41	3.95 ± 0.38	4.64 ± 0.19	4.89 ± 0.22
Met	1.09 ± 0.08	1.44 ± 0.18	1.39 ± 0.41	1.76 ± 1.02	1.19 ± 0.09	1.62 ± 0.32
Ile	2.75 ± 0.36	3.85 ± 0.22	2.08 ± 0.39	2.32 ± 0.26	2.75 ± 0.27	2.93 ± 0.39
Leu	7.75 ± 0.78	7.73 ± 0.23	6.09 ± 0.33	6.11 ± 0.39	7.75 ± 0.28	7.19 ± 0.44
Tyr	8.15 ± 0.10	*6.01 ± 0.09	13.69 ± 0.60	**9.83 ± 1.31	7.02 ± 0.47	*5.14 ± 0.35
Phe	3.11 ± 0.07	**2.79 ± 0.07	5.32 ± 0.13	*4.33 ± 0.16	2.75 ± 0.07	**2.45 ± 0.12

Fig. 1. Scans of polyacrylamide gels at 280nm obtained after electrophoresis of equal weights of the soluble fibril fraction (g), derived from normal mouse hair (□) and from Naked-mouse hair (■)

The amount (mol%) of glycine and of tyrosine present in the protein fractions eluted from both gels (in the regions between the arrows) are as follows:

	(I)	(II)	(III)
Control			
Glycine	30.6	6.6	8.6
Tyrosine	10.6	1.0	Trace
Naked			
Glycine	30.0	6.1	8.9
Tyrosine	8.4	1.3	1.0

The horizontal arrow shows the direction of migration and the peak labelled BPB corresponds to Bromophenol Blue.

of these extracts, which contains the HG proteins, was then subjected to polyacrylamide-gel electrophoresis. The same 12 peaks were obtained, indicating that no new peaks arose with increasing times of reduction.

Discussion

The results point to three major conclusions. (1) The abnormal amino acid composition of Naked-mouse hair is largely the result of a deficiency of a protein fraction which is rich in glycine and tyrosine.

Fig. 2. Elution diagram of S-carboxymethylkerateine extracted from normal (□) and from Naked (■) mouse hair on Sephadex G-75

To the column (90cm×2cm) were applied 30.8mg (26.1 E_{280} units) of normal S-carboxymethylkerateine and 30.0mg (27.8 E_{280} units) of Naked-mouse S-carboxymethylkerateine. The proteins were eluted with a buffer of 0.1M-sodium phosphate/6M-urea, pH7. The amount (mol%) of glycine and tyrosine in the fractions pooled from normal and Naked-mouse S-carboxymethylkerateine are as follows:

	1	2	3	4
Control				
Glycine	6.8	14.9	29.1	32.6
Tyrosine	2.4	5.2	16.6	20.0
Naked				
Glycine	6.8	10.8	22.4	25.5
Naked	2.3	3.2	10.6	13.5

(2) From our data, we calculate that hair from Naked mice contains approximately one-half of the normal amount of this protein fraction (see below). (3) The deficiency involves more than one molecular species.

Deficiency of HG protein

Table 1 shows that hair from Naked mice and all the fractions extracted from it, with the exception noted above, are markedly and significantly deficient in tyrosine and glycine. Further, other amino acid differences in extracted fractions are consistent with the loss of HG proteins. On the other hand, the insoluble residue, which remains after extraction, contains very similar amounts of glycine and tyrosine. These observations are consistent with the fact that the HG proteins are known to be extracted by the methods used (Gillespie, 1972).

Since the HG proteins are all present in the extract,

the proportion of Naked-mouse hair which is extractable should be abnormally low. Regression analysis showed that this is the case (see Table 2). Moreover, we found that the deficiency resides largely in the soluble fibril fraction, which is known to be particularly rich in HG proteins (Gillespie, 1972).

Polyacrylamide-gel electrophoresis of the soluble fibril fraction and gel filtration of S-carboxymethylkerateine show that those proteins in which Naked-mouse hair is deficient are of the HG type, whereas those proteins that are present in similar amounts in both genotypes do not belong to this class (Figs. 1 and 2).

Magnitude of deficiency

The proportion of HG protein missing from Naked-mouse hair may be calculated from the data in three independent ways.

(a) Table 2 shows that Naked-mouse hair contains 10% less extractable protein than normal hair, and therefore 10% less HG protein, assuming that the loss of soluble protein is entirely the result of HG protein deficiency.

(b) Gillespie & Frenkel (1974) have shown, as the result of studying samples of keratin derived from a large number of species, that the percentage of HG protein present in keratin is an almost precisely linear function of its tyrosine content. The equation of the line depicted by the authors, though not stated by them, is $y = 5(x - 2)$, where y is the percentage of HG protein present in the keratin and x is its tyrosine content (in mol%).

Our values for the tyrosine contents of normal and Naked-mouse hair are 5.18 and 3.65 mol% respectively. Substituting in the above equation, these values would give an HG content of 16 and 8% for normal and Naked-mouse hair respectively, representing a loss of one-half of the normal complement of HG proteins. The differences in the heights of the electrophoretic peaks (Fig. 1) and of the low-molecular-weight peaks (Fig. 2) are also of this order of magnitude. It is dangerous to rely too much on this, however, since differences of extinction coefficient have to be taken into account.

(c) We can calculate by regression analysis [see the Appendix (Gold *et al.*, 1976)], how much HG protein of known composition (Gillespie, 1972) would have to be removed from normal hair to give the amino acid composition observed in Naked-mouse hair. This calculation produces a value of 12%.

Considering the inaccuracies inherent in the assumptions on which these calculations are based, the agreement achieved by the three methods is reasonable.

Loss of several molecular species

Gillespie (1972) has shown that the HG proteins are highly heterogeneous, comprising at least 20, and probably more, different kinds of protein. It is very unlikely therefore that the loss of any one of them could result in a change of amino acid composition of the magnitude observed. Our results support this conclusion.

Gel electrophoresis resolved the fibril soluble fraction into several proteins, which were present in lesser amounts in Naked-mouse hair. Proteins recovered from two of these peaks proved on analysis to be of the HG type.

When the component of S-carboxymethylkerateine of lowest molecular weight, having an amino acid composition of the HG type, was subjected to polyacrylamide-gel electrophoresis, 12 components were resolved. This heterogeneity was not the result of the extraction procedure, since no new proteins appeared when the time of extraction was varied.

Densitometry showed that none of the 12 constituted a sufficient proportion of the total such that the loss of it alone would account for the observed deficiency in the whole fraction.

Mode of gene action

It is not possible to decide, on the basis of our present data, whether the loss of HG protein is a proximate consequence of the Naked mutation. That is to say, we do not know whether the gene at the mutant locus is directly involved in the production of these proteins, or whether, for example, the mutation causes a much more generalized tissue malformation of which the loss of HG protein is merely a reflexion. In this connexion, one must bear in mind that the insoluble residue derived from Naked hair is abnormal in amino acid composition (Table 1). For this reason, the loss of HG protein is unlikely to be the only change caused by the Naked mutation. The other changes may represent an alteration in hair architecture secondary to the loss of HG protein.

Distribution of HG proteins

The significant decrease in the glycine and tyrosine content of the matrix proteins from the mutant hair provides a striking experimental confirmation of the theoretical conclusion of Fraser *et al.* (1973), that some of the HG proteins must reside in the matrix.

We thank Dr. F. Clarke Fraser for providing the mice, Ms. Rhea Bayreuther for technical assistance, Dr. Peter Hechtman, Dr. Gerald Lancaster, Dr. Terry Stacey and Ms. Linda Stirk for critical review of the manuscript, and Ms. Lynne Merid and Ms. Huguette Ishmael for assistance in preparing it. This work was supported by a grant from the Medical Research Council of Canada to its Genetics Group.

References

- David, L. T. (1932) *Z. Zellforsch. Mikroskop. Anat.* **14**, 616-719
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404-427
- DeDeurwaerder, R. A., Dobb, M. G. & Sweetman, B. J. (1964) *Nature (London)* **203**, 48-49
- Fraser, R. D. B., Gillespie, J. M. & Macrae, T. P. (1973) *Comp. Biochem. Physiol. B* **44**, 943-947
- Gillespie, J. M. (1972) *Comp. Biochem. Physiol. B* **41**, 723-734
- Gillespie, J. M. & Frenkel, M. J. (1974) *Comp. Biochem. Physiol. B* **47**, 334-346
- Gold, R. J. M. & Kachra, Z. (1974) in *Proc. Hair Symp. 1st, Atlanta, GA* (Brown, A. C., ed.), pp. 260-276, Medcom Press, New York

- Gold, R. J. M. & Scriver, C. R. (1971) *Proc. Conf. Clinical Delineation of Birth Defects 3rd*, vol. 7, pp. 91-95
- Gold, R. J. M. & Scriver, C. R. (1972) *Am. J. Hum. Gen.* **24**, 549-561
- Gold, R. J. M., Tenenhouse, H. S. T. & Adler, L. S. (1976) *Biochem. J.* **159**, 157-160
- Greenland, R. D., Roth, W., Gieske, T. & Michaelson, I. A. (1974) *Anal. Biochem.* **62**, 305-309
- Lebedinsky, N. G. & Dauvart, A. (1927) *Biol. Zentralbl.* **47**, 748-752
- McKusick, V. A. (1971) *Mendelian Inheritance in Man, Catalogs of Autosomal Dominant, Autosomal Recessive, and X-linked Phenotypes*, 3rd edn., Johns Hopkins Press, Baltimore and London
- Scriver, C. R., Solomons, C. C., Davies, E., Williams, M. & Bolton, J. (1965) *J. Pediat.* **67**, 946-947
- Steinberg, A. G. & Fraser, F. C. (1946) *Can. J. Res. Sect. D* **24**, 1-9
- Tenenhouse, H. S., Gold, R. J. M., Kachra, Z. & Fraser, F. C. (1974) *Nature (London)* **251**, 431-432

APPENDIX

A Method for Calculating the Relative Protein Contents of the Major Keratin Components from their Amino Acid Composition

By REYNOLD J. M. GOLD,* HARRIET S. TENENHOUSE* and LEONDA S. ADLER†

*The deBelle Laboratory for Biochemical Genetics, McGill University, Montreal Children's Hospital Research Institute, 2300 Tupper Street, Montreal, Quebec H3H 1P3, Canada, and

†Concordia University, Montreal, Quebec, Canada

(Received 8 April 1976)

In the main paper (Tenenhouse & Gold, 1976), we described the separation of keratin fibres into major components whose amino acid composition were determined. We also wished to know the relative amounts of protein in each component. We have developed a new method for calculating these amounts solely from the amino acid composition without the necessity for any further experimental data.

Here, we explain the theoretical basis of the method, discuss its reliability under experimental conditions, compare it with other available methods and consider applications of the method to further problems.

Theoretical Basis

If a mixture of proteins containing t mol% of a given amino acid is separated into an insoluble and a soluble fraction containing respectively i and s mol% of the same amino acid, and if the insoluble fraction contains a proportion, p , of all amino acid residues present in the original mixture, then

$$t = pi + (1 - p)s \quad (1)$$

or

$$p = \frac{t - s}{i - s} + 0 \quad (2)$$

This equation will be true for any amino acid chosen. Therefore, if $t-s$ is represented on the y axis

and $i-s$ on the x axis, the point representing each amino acid should lie on a straight line, passing through the origin, and whose slope is p . The slope of the line thus gives the proportion of amino acid residues in the insoluble fraction.

Experimental error will scatter the points from this theoretical line, but β , an estimate of p , can be arrived at by regression analysis, according to the following expression

$$\beta = \frac{\sum_{k=1}^n x_k y_k}{\sum_{k=1}^n (x_k)^2} \quad (3)$$

where x_k and y_k are given by $i-s$ and $t-s$ respectively for the k th amino acid, and n amino acids are considered. β is then the least-squares estimate of p , with the constraint that the line must pass through the origin.

Further, s_β the standard deviation of β , is given by

$$s_\beta = \frac{\sum_{k=1}^n (y_k - x_k \beta)^2}{\sum_{k=1}^n (x_k)^2} \cdot \frac{1}{(n-1)} \quad (4)$$

Finally, if a protein mixture is separated into several fractions, the problem can be solved for all fractions simultaneously by multiple linear regression.