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Capillary electrophoresis of hair proteins modified by alcohol intake in laboratory rats

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Abstract

A capillary zone electrophoretic method was used to obtain profiles of solubilized rat hair keratin proteins. The same methodology was used to reveal the presence of additional protein peaks in alcohol-consuming rats. Two types of separation were investigated. Alkali-solubilized keratins from hair of rats treated for 5 weeks with 5% ethanol and 2 weeks with 10% ethanol (instead of drinking water) and from controls were analysed. Whereas under alkaline conditions (pH 9.2, 50 m*M* borate) an additional fraction of "low-sulphur" keratins with the highest anodic mobility of this keratin category was shown in alcohol-treated animals, acid electrophoresis carried out at pH 3.5 in phosphate buffer (50 m*M*) revealed the presence of two sharp peaks absent in the controls. These findings were confirmed by two-dimensional separations of carboxymethylated keratin samples. An attempt was made to identify further one of the newly occurring fractions in alcohol-consuming animals. It was revealed that the tryptic hydrolysate of "low-sulphur" proteins obtained from alcohol-consuming animals contained a peptide not found in controls.

1. Introduction

Hair analysis is gaining considerable popularity in situations where the past history of drug abuse rather than the present state needs to be investigated [1,2]. Whereas morphine, cocaine and a number of other drugs can be extracted from hair after acid, alkaline or solvent treatment of the matrix (for reviews, see Refs. [3 and 4]) (the nature of the way of binding these drugs to the hair matrix remains unknown), apparently there are other metabolites that can interact with the hair keratins. In a previous paper we showed that profiling of the organic phase-extractable material from acid-treated hair may be used as a diagnostic tool in animals predisposed to alopecia areata [5]. Biochemical studies of wool filaments and their associated matrix material have led to the realization that there are three major classes of hair proteins: the low-sulphur keratins (designated 40–60 K), the high-sulphur keratins (designated 10–25 K) and the high glycine–tyrosine proteins (designated 10–25 K). The analysis of solubilized hair keratins by standard gel electrophoresis [6] reveals two fractions (one doublet and one triplet) of probably low-sulphur keratins in the relative molecular mass (M_r) regions of $44/46 \cdot 10^3$ and of $56/59/60 \cdot 10^3$. In the case of keratin characterization by two-dimensional gel electrophoresis, six low-sulphur (M_r 55 500–76 000) and seven high-sulphur (M_r 26 500–43 000) major proteins [7] were

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found. It is likely that the molecular masses of these fractions are higher than the real values. Although, as shown also in this report, two-dimensional gel electrophoresis offers valuable separations in profiling hair keratins, capillary zone electrophoresis (CZE) appears more rugged and faster and offers complementary information.

The idea behind investigating hair keratin of alcohol-treated animals is that long-term alcohol intake may cause metabolic alterations that could be expressed and accumulated in long-lived proteins; the production of acetaldehyde (the first metabolite of ethanol) should be the first to be considered. It has been reported that ethanol administration leads to free amino group modification in this way [8]. The objection that the amount of acetaldehyde released is too small to cause any alterations in long-lived proteins should be abandoned not only because of the results just mentioned [8], but also because our experience indicates [9] that even minute concentrations of oxo-containing compounds lead to protein modifications that can be demonstrated, e.g., by decreased solubility.

In this work, we investigated the possibility of separating hair keratins by capillary electrophoresis and attempted to show the differences in keratin protein profiles in hair of rats consuming alcohol.

2. Experimental

2.1. Animals

Two sets of male Wistar rats were housed three per cage (twelve altogether) and fed ad libitum a standard pelleted diet. They were taken into the experiment when 60 days old. Alcohol was administered by replacing water with 5% ethanol solution for 35 days and with 10% ethanol solution for the following 14 days. After this time period the hair from the dorsal region was shaved (about 2.2 g) and subjected to further analysis. The same number of animals (two cages, three animals each) kept on tap water served as controls. The alcohol-treated animals did not differ in the growth curve in comparison with the controls, but the liquid and food intake was decreased in the experimental group, representing 71% and 78% of the controls (at the end of the experiment), respectively.

2.2. Preparation of samples

The hair sample (about 2.2 g) taken from the dorsal part of the experimental animal was washed stepwise with ethanol and 0.01 *M* phosphate buffer (pH 6.0) on a Büchner funnel. Two approaches for solubilizing hair proteins were used. At the beginning we used incubation (1.1 g) with 1 *M* NaOH (125 ml) at 40°C overnight, as is the procedure often used in forensic analyses [10]. The insoluble residue was filtered and the solubilized fraction was extracted three times with 125 ml of chloroform–2-propanol (9:1, v/v) (the aqueous phase was injected). At a later stage, hair samples after the Büchner funnel wash were minced into 2–3-cm pieces and incubated under nitrogen in 8 *M* urea–200 m*M* Tris–HCl buffer (pH 9.5)–2 m*M* mercaptoethanol for 2 h. After this, the swollen hair samples were homogenized and the incubation was continued for a further 2 h at 40°C. The insoluble residue was spun off (10 000 *g*, 10 min) and the supernatant was stored frozen in small aliquots until used. As the profiles obtained by these two approaches did not differ, the latter procedure was mostly used. For two-dimensional gel separations, the dissolved proteins in 50-μl aliquots were radiolabelled at pH 8.0 by S-carboxymethylation of a proportion of the cystein residues with [2-^{14}C]iodoacetic acid (6 μCi). After 10 min at room temperature, excess iodoacetate was added to complete the modification of the cysteine residues. Excess iodoacetate remaining after 10 min was reacted with 2-mercaptoethanol [11,12].

2.3. Capillary zone electrophoresis (CZE)

The electrophoretic separations were carried out in a 50-μm fused-silica capillary (Polymicro Technologies, Tucson, AZ, USA) mounted in a Model 350, CZE apparatus (ISCO, Lincoln, NE, USA) run routinely at 15 kV (45 μA) with UV detection at 220 nm. Separations were per-

formed under both alkaline and acidic conditions with two kinds of buffers. For the alkaline runs, 50 m*M* borate buffer (pH 9.2) was used, whereas the acidic separations were run in 50 m*M* phosphate buffer (pH 3.5). The capillary was occasionally rinsed with 0.1 *M* NaOH (by aspiration), whereas it was rinsed with the run buffer before each run. Routinely, 7 μl of samples were injected through a 1:1000 split-stream port with an HPLC-type syringe into the ISCO apparatus. Samples were sonicated for 15 min prior to analysis, diluted appropriately (see Results) and filtered through a Millex HV 0.45-μm filter (Waters–Millipore, Milford, MA, USA). Omitting these operations leads at least to a noisy baseline or may spoil the run completely.

The acidic runs were much slower than under alkaline conditions, owing to the large difference in the electroosmotic flow. The positions of individual peaks varied very little (less than 5% R.S.D. of the retention time, except for the slow peaks in the acidic, long-lasting runs). Alkali-solubilized hair from alcohol-treated animals and alkali-solubilized hair from controls were subjected to analysis.

2.4. Polyacrylamide gel electrophoresis

Radiolabelled protein extracts were examined by two-dimensional polyacrylamide gel electrophoresis. Two systems were used in the first dimension separation, being different in each case. Protein separation was carried out in 8 *M* urea at pH 8.9 or pH 3 in an apparatus with glass tubes (130 × 3.0 mm). The second-dimension separation was according to the apparent M_r by sodium dodecyl sulphate (SDS) electrophoresis in a slab (140 × 120 × 1.5 mm).

For the separation at pH 8.9, the procedure of Davis [13] was used, except that 8 *M* urea was incorporated into the gels. The proteins (10 μl of protein extract) were separated on polyacrylamide gel rods consisting of 10-mm 4% stacking gel and 100-mm 7.5% separation gel. These gels were prepared from a stock 29.2% acrylamide–0.8% N,N′-methylenebisacrylamide solution, and polymerized using ammonium peroxodisulphate. The electrophoresis was run at 40 V for approximately 18 h until bromophenol blue tracking dye (loaded on a similar gel rod or added to the upper electrode buffer) had travelled the length of the gel rod.

For electrophoresis at pH 3, the system reported by Marshall and Gillespie [12] was used. The proteins (5 μl of protein extract and 10 μl of acid sample solution) were separated on 110 × 3.0 mm 8% polyacrylamide gel rods [acrylamide–N,N′-methylenebisacrylamide (27:1)] containing 4.7 *M* acetic acid and 8 *M* urea. Electrophoresis was performed towards the cathode at 80 V until the crystal violet tracking dye had travelled the length of the gel rod (approximately 18 h).

SDS electrophoresis was carried out according to Laemmli [14] and O'Farrell [15]. Polyacrylamide gel slabs were prepared from a stock 29.2% acrylamide–0.8% N,N′-methylenebisacrylamide solution and polymerized using ammonium peroxodisulphate. A stepwise acrylamide concentration was used by Marshall and Gillespie [12]. The lower 20 mm of the separation gel slab consisted of 15% polyacrylamide and the upper 75 mm 10% polyacrylamide. The first-dimension gel rod was held in place above the 20-mm stacking gel (4.5% polyacrylamide) by 1% agarose [15]. Gel rods after electrophoresis at pH 3 were equilibrated for 45 min with the SDS sample buffer (buffer 0 in Ref. [12]), but this equilibration step was not necessary after isoelectric focusing or pH 8.9 electrophoresis. Electrophoresis was performed at 100 V for 1 h, then at 200 V until the bromophenol blue tracking dye (added to upper electrode buffer before commencement of electrophoresis) had travelled about 10 mm through the 15% polyacrylamide gel. Full details of the procedures were given by Marshall and Gillespie [12].

After SDS electrophoresis, radiolabelled proteins were located by fluorography [16].

2.5. Ion-exchange separation of solubilized keratins

Ion-exchange separation was carried out by passing 5 ml of the supernatant containing alkali-solubilized keratins through a Bio-Rad (Richmond, CA, USA) AG 50W-X8 filter, the cation-exchange bead was flushed with distilled water (5

ml) and the retained fraction was eluted with 0.1 *M* triethylamine (5 ml). After evaporation (at 50°C in vacuo), the residue was reconstituted in 2 ml of Milli-Q-purified water and after overnight dialysis the sample was ready for further analysis.

2.6. Trypsin digestion

The retained fraction from the Bio-Rad AG 50W-X8 cartridge was prepared as described above except that the dry residue was reconstituted in 50 m*M* NH_4HCO_3–1 m*M* $CaCO_3$. Trypsin solution was prepared in the same buffer and the sample was digested at an enzyme-to-protein ratio of 1:10 at 37°C overnight. After stopping further hydrolysis, the digest was either used directly for reversed-phase chromatography or, after drying on a Speed Vac Centrifuge, used for mass analysis.

2.7. HPLC of the trypsin-released peptides [17]

The peptides were separated on a reversed-phase column (Vydac C_{18}, 7 μm, 250 × 0.42 cm I.D.). A linear gradient from 100% A to 70% B within 1 h was applied, where A = 0.10% trifluoroacetic acid (TFA) in water and B = 0.10% TFA in 70% acetonitrile. The flow-rate was 1 ml/min. Collected fractions containing peptides were taken to dryness in a Speed Vac centrifuge.

2.8. Plasma desorption mass spectrometry (PDMS) [18]

Peptides separated by reversed-phase HPLC were dissolved in 8 μl of 0.1% TFA–20% methanol and applied to nitrocellulose-covered targets. Targets were then washed in 10 μl of water (UHQ) to remove salt and PDMS was performed on a Model 20 plasma desorption mass spectrometer (Biolon, Uppsala, Sweden). Each spectrum was accumulated for 10^6 fission events using an acceleration voltage of 15 kV and calibrated with H^+ and NO^+ ions.

3. Results

In contrast to acid extraction [5], alkaline treatment solubilizes much of the hair matrix (about 85%). For capillary electrophoretic analysis, the protein concentration had to be adjusted prior to analysis (50–100 μl to 5 ml of Milli-Q-purified water).

Typical electropherograms of the rat hair keratin fractions obtained from both alcohol-treated and control animals are shown in Fig. 1, where the results are compared with both one- and two-dimensional electrophoresis. Two groups of peaks are seen in the capillary separations: the first group (two peaks in controls, three peaks in alcohol-treated animals) corresponds to the fast-moving zone in plate A_1. The other set of peaks moving with retention 7.5–10 min and two additional peaks moving with retention 15 and 18 min represent the other (smearing zone) seen in one-dimensional electrophoresis.

In two-dimensional gel separations, two categories of protein spots can also be seen, a fast, cathodically moving set of keratin proteins and a long diagonal smear. In accordance with literature data, the two fast-moving peaks in extracts from controls (three in alcohol-consuming rats) were tentatively identified as "low-sulphur" keratins, whereas the rest corresponding to the diagonal smear were assumed to represent the "high-sulphur" keratin proteins.

By passing the keratin protein extract through the Bio-Rad cation-exchange filter, both categories of hair proteins can be separated: the "low-sulphur" proteins are retained whereas the "high-sulphur" proteins are not, as can be seen from Fig. 2; the retained fraction (released by washing the filter with 2 ml of 0.1 *M* triethylamine, followed by overnight dialysis of the filtrate) corresponds to the first two (or three in alcohol-treated animals) fractions appearing during the capillary electrophoretic runs (see Fig. 2, plates A and C). The unretained proteins correspond to the remaining, more anodically moving peaks. Amino acid analysis of both fractions (data not presented) indicated a higher proportion of sulphur-containing amino acids in the

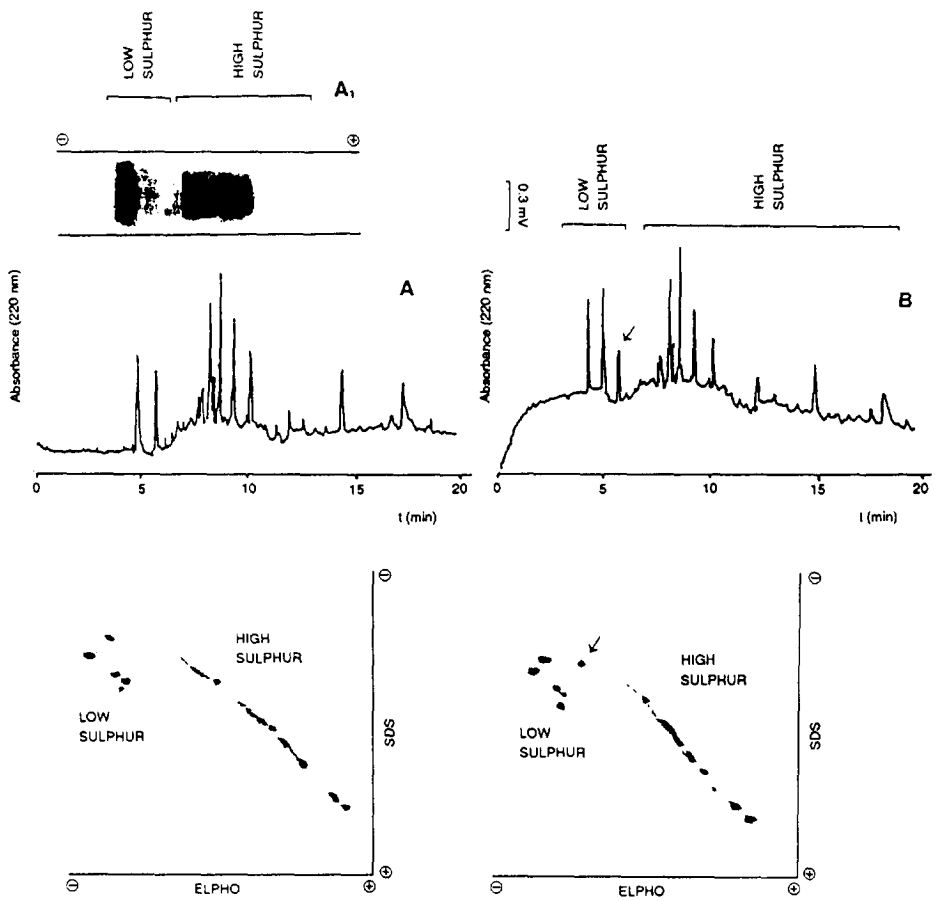

Fig. 1. Comparison of capillary electrophoretic profiles (pH 9.2) of hair keratin samples obtained from (A) control rats and (B) alcohol-treated rats. The dilution of extracts was 50 μl to 5 ml. The position of the peak present in the alcohol-treated animals but absent in controls is indicated by an arrow. Assignment of "high-sulphur" and "low-sulphur" proteins is based on the results shown in Fig. 2. CZE conditions as specified under Experimental.

unretained fraction, justifying the classification of the two sets of protein fractions as "low-sulphur" and "high-sulphur" types.

From the profiles shown in Fig. 1, it is evident that the profiles obtained from controls (plate A) and alcohol-treated animals (plate B) differ in the arrow-indicted peak. The additional fraction belongs, according to its electromigration behaviour, between the "low-sulphur proteins" and has an apparent M_r of $70 \cdot 10^3$.

Further investigation of the "high-sulphur" proteins by two-dimensional electrophoresis under alkaline conditions was not possible owing to the smearing character of the "high-sulphur protein" zone. Therefore, separations run at pH 3.5 as visualized in Fig. 3 were run. These indicated the presence of two spots in the M_r $25 \cdot 10^3$–$30 \cdot 10^3$ region in two-dimensional electrophoresis. In CZE separations (Fig. 3, plates A and B) run at pH 3.5, two distinct peaks appeared at run times between 10 and 20 min in alcohol-treated animals (indicated by arrows) which were absent in control runs. When the fraction retained on the Bio-Rad AG 50W-X8 filter was run under similar conditions, the picture shown for plate C was obtained. By comparison of plate C with plates A and B, the peaks moving at 12 min and less in CZE can be identified as "low-sulphur" and those moving more rapidly to the anode represent the "high-

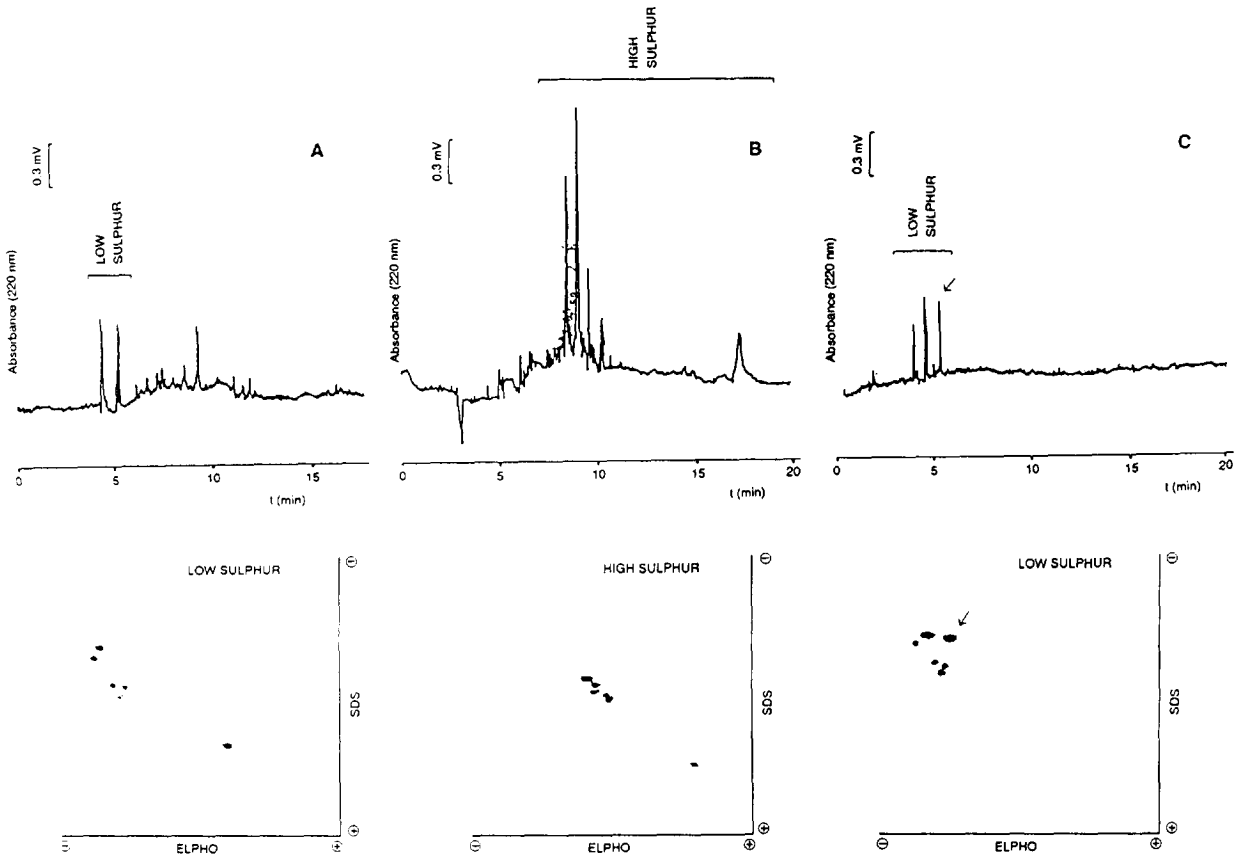

Fig. 2. CZE profiles (pH 9.2) of the retained (plate A) and unretained (plate B) fractions obtained after passing the crude extract through a Bio-Rad AG 50W-X8 filter. "High-sulphur" proteins were recovered in the filtrate whereas "low-sulphur" proteins were released by 0.1 *M* triethylamine. Comparison with the two-dimensional gel separations is visualized under each CZE run. The position of the fraction of "low-sulphur" proteins occurring in alcohol-treated animals but absent in controls is indicated by an arrow. Profile of proteins retained on the cation-exchange cartridge from the alcohol-treated rat hair preparation is shown on plate C. CZE conditions as specified under Experimental.

sulphur" fraction. It appears that of the three additional peaks occurring with in animals treated for 7 weeks with alcohol, two belong to the category of "high-sulphur" proteins whereas the third fraction can be traced within the "low-sulphur" protein fraction (alkaline separation conditions).

The easy separation of the "low-sulphur" fraction containing an extra peak in alcohol-treated rats (Fig. 1, plate A), the presence of which was confirmed by two-dimensional electrophoresis, prompted us to attempt to characterize further the "low-sulphur" keratin fraction observed in alcohol-treated animals. The Bio-Rad AG 50W-X8-retained fraction was routinely eluted with 0.1 *M* triethylamine, lyophilized and hydrolysed with trypsin as described under Experimental. The resulting mixture of peptides was separated by reversed-phase chromatography (Fig. 4). This separation resulted in a very complex mixture, in which 23 peaks could be discerned. All of these were collected, taken to dryness and the residue dissolved in 8 μl of 0.1% TFA–20% methanol and investigated by PDMS. Of the 23 fractions, 11 gave non-interpretable spectra (numerous masses). Peak 11 with M_r 1217.6, however, was present in the preparations obtained from alcohol-treated animals only (Fig.

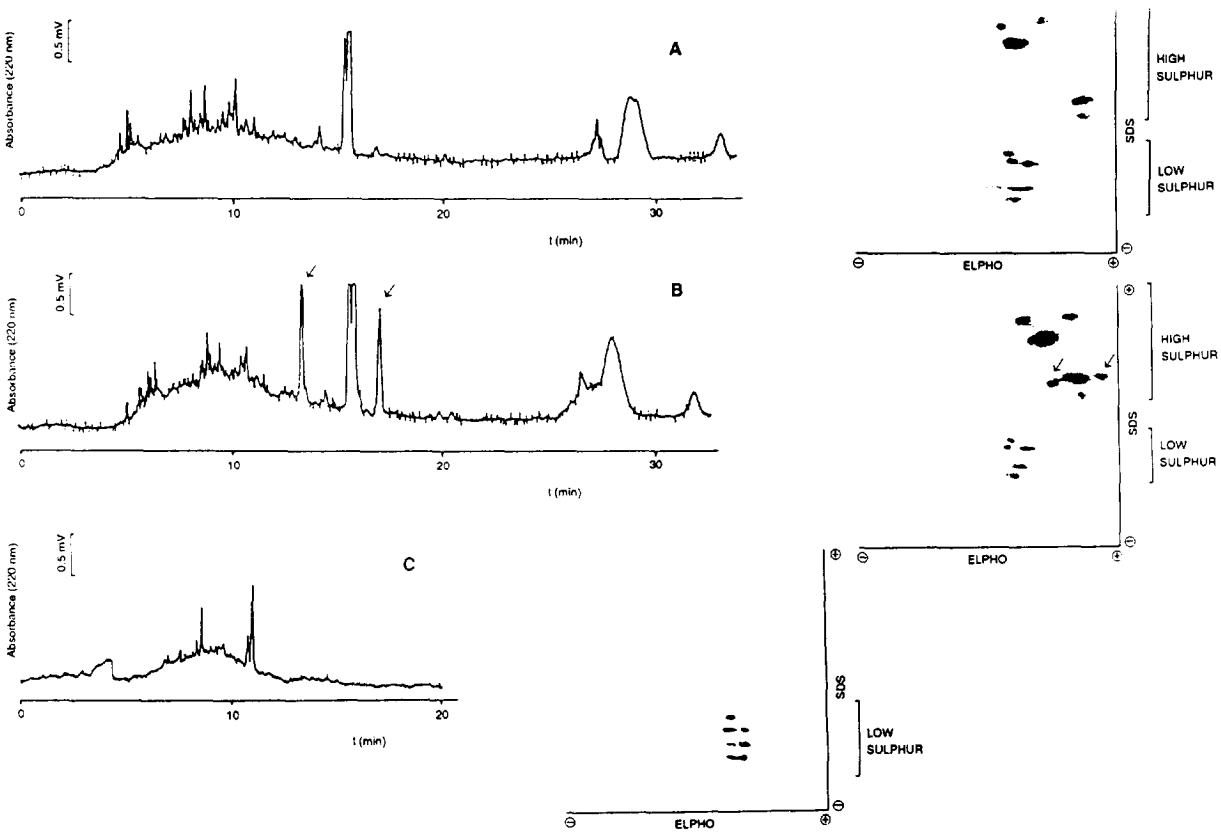

Fig. 3. CZE profiles and two-dimensional electrophoresis of rat hair extracts under acidic conditions. CZE run at pH 3.5, two-dimensional separation at pH 3.0 (see Experimental for details). Plate A, controls; plate B, alcohol-treated, 7 weeks; plate C, fraction of control animals retained on the Bio-Rad AG 50W-X8 filter and released with 0.1 *M* triethylamine, corresponding to "low-sulphur" proteins.

5) and was absent in preparations obtained from controls.

4. Discussion

CZE can be used not only for the analysis of organic phase-extractable material from hair, as previously reported [5], but also for the analysis of hair proteins. Reproducible profiles were obtained with less than 5% R.S.D. of the retention time on both inter-run and day-to-day bases. The capillary electrophoresis profiles of hair keratins can be separated into two parts: (i) the set of peaks appearing soon before the detector window (two in controls, three in alcohol-consuming rats), which in accordance with their behaviour in two-dimensional electrophoresis correspond to "low-sulphur" keratins, and (ii) the peaks in the remaining part of the electropherogram, which according to the same comparison could be tentatively identified as "high-sulphur" proteins. These two categories can be easily separated by passing the extract through a cation-exchange cartridge on which the more basic set of peaks is retained. Analysing the filtrate and retentate for the content of sulphur-containing amino acids indicated considerably more (about twice per amino acid nitrogen) sulphur-containing amino acids in the unretained fraction.

In the category of "low-sulphur" proteins, one additional peak in CZE and one additional spot in two-dimensional gel electrophoresis was ob-

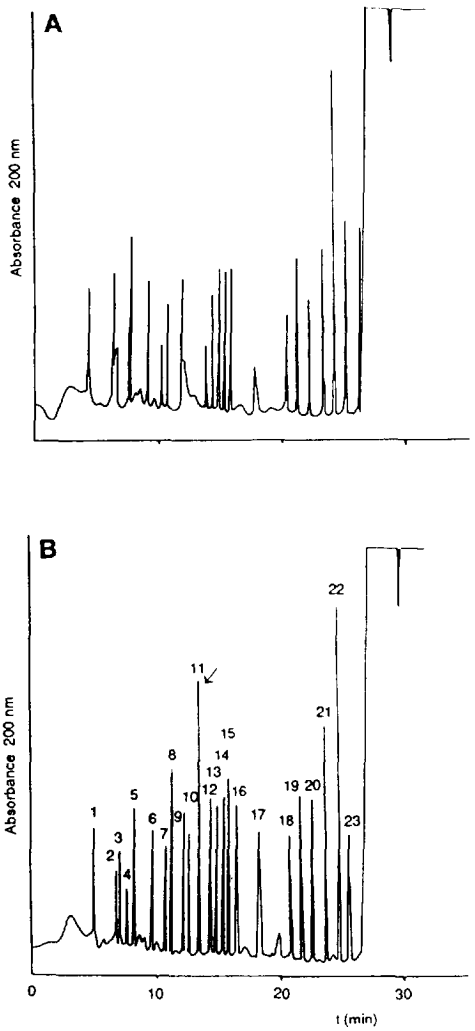

Fig. 4. HPLC of the trypsin hydrolysate of the "low-sulphur" keratin fraction. For preparation, see Experimental. (A) controls; (B) alcohol-treated rats. Note the absence of peak 11 (see arrow) in controls.

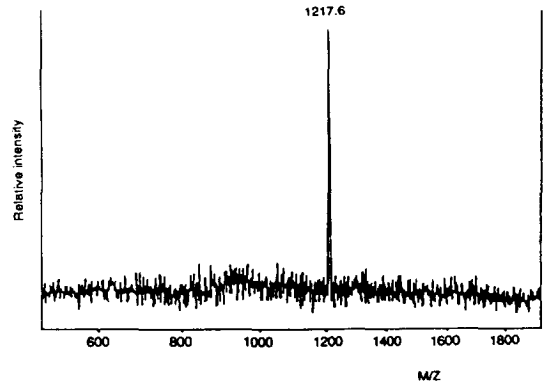

Fig. 5. PD mass spectrum of the HPLC fraction present in the alcohol-treated animals only (peak 11, Fig. 4B).

served. This protein fraction exhibited the highest anodic mobility of the proteins constituting the "low-sulphur" category and, according to the gel electrophoretic behaviour, possessed an apparent M_r of $70 \cdot 10^3$. When the separations were run at acidic pH, the profiles of controls and treated animals differed in two peaks (zones) present in the "high-sulphur" region both in the CE runs and in two-dimensional gel electrophoresis.

Fractionation of peptides obtained of trypsinization of the "low-sulphur" proteins indicated the presence of a peptide of M_r 1217.6, which was absent in preparations obtained from untreated animals.

The nature of the protein fractions occurring in alcohol-treated animals should reflect either metabolic changes or post-translational modifications of normally synthesized proteins, or both. To express a definite conclusion in this respect is difficult at present, although some idea can be obtained from the MS data. The peptide found in the "low-sulphur" protein trypsin hydrolysate of alcohol-treated animals was not found in controls. Whether this reflects the presence a metabolite-modified protein is difficult to say.

The fact that it was possible to detect three additional protein fractions in alcohol-treated animal hair keratin preparations appears to indicate a polytopic effect of alcohol consumption on hair keratin. Although no proof has been obtained that these additional fractions represent three different proteins, it seems to be so as the additional "low-sulphur" fraction may not be revealed under acidic separations and, on the other hand, the two "high-sulphur" protein fractions may not be revealed alkaline conditions of electrophoresis (both CE and two-dimensional gel electrophoresis).

From the separation point of view, CZE may serve as an additional tool in separating keratin proteins. This paper represents, to our knowledge, the first attempt in this respect. Although

comparison of carboxymethylated and untreated proteins (i.e., the results of two-dimensional electrophoresis and CZE) may be the subject of discussion because of the structural changes introduced into the protein molecule by carboxymethylation, here we attempted to categorize the peaks occurring in the CZE separations according to a recognized separation procedure.

5. Conclusions

CZE was applied to a category of proteins not previously investigated by this method. Two basic categories of alkali-extractable keratins, namely the "high-" and "low-sulphur" fractions, were easily separated, each of these categories exhibiting multiple peaks. Comparison with two-dimensional electrophoresis revealed lower selectivity, e.g., in the region of "low-sulphur" proteins (five zones separated by two-dimensional gel electrophoresis while only two peaks were obtained by CZE). This, however, may be caused by the fact that in CZE underivatized extracts were used whereas in the two-dimensional gel separations the samples of proteins were carboxymethylated. Certainly further investigations are needed to make CZE generally applicable to the separation of keratins. On the other hand, CZE was capable of revealing the differences in keratin protein profiles caused by alcohol intake over a period of 7 weeks in laboratory rat hair. The differences regarding the number of peaks in which the individual profiles differed were confirmed by two-dimensional gel electrophoresis. It can be concluded that CZE profiling of rat hair keratins is capable of discerning three peaks that occur only in alcohol-consuming animals, whereas no such peaks were obtained for preparations obtained from controls.

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