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CHROMATOGRAPHY OF INORGANIC IONS ON THIN LAYERS OF PROTEIN

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SUMMARY

The preparation of thin layers of natural and chemically modified keratin from wool cortical cells is described. The R_F values of a number of cations and anions on these thin layers have been measured. The migration of these ions is interpreted in terms of ion exchange with the protein substrate.

INTRODUCTION

There has been considerable interest in the ion-exchange chromatography of inorganic ions on both inorganic and synthetic organic substrates of various types. The purpose of this work is to describe the chromatography of a number of cations and anions on thin layers of a naturally occurring protein, wool keratin, and to demonstrate the way in which the chromatographic behaviour of this material may be altered by chemical modification.

In its natural state, wool is a complex protein which contains a number of chemically reactive groups; these include hydroxyl, amino, amide, carboxyl, sulphydryl and disulphide groups. The amino groups are derived from arginine, lysine and histidine and the carboxyl groups from glutamic and aspartic acids. By titration and analysis, wool has been shown to have acid and base binding capacities both of about 0.8 mequiv./g[1]. These capacities compare favourably with those of many synthetic ion-exchange resins and it might be expected that wool keratin should exhibit the properties of an amphoteric (weak acid, weak base) ion exchanger. Wool has in fact been shown to have ion-exchange capability[2] but because of its fibrous form, it has not been especially convenient or effective for use in chromatographic procedures. Recently, wool has been prepared in a form more suitable for chromatography by rendering fibres into component cortical cells; these are spindle shaped cell residues (approx. 100 × 4 μm) consisting almost entirely of insoluble protein and thin layers and columns have been prepared from them[3]. Since wool can be readily esterified and deaminated, it is also possible to prepare thin layers of cortical cells which exhibit predominantly weak base or weak acid ion-exchange capability.

Previously, cations and anions have been separated on thin layers of resin

granules bound with cellulose and calcium sulphate[4] and on paper that had been impregnated with finely powdered ion-exchange resins[5]. Inorganic exchangers, such as insoluble salts of the tetravelent metals zirconium and titanium, have also been used both as thin layers[6] and impregnated papers[7]. Again, paper containing absorbed liquid anion exchangers has been employed for the separation of cations[8]. While unmodified cellulose is of little value as an ion exchanger because of its low capacity[9] considerable use has been made of modified cellulose containing, in particular, strong acid or basic groups. The only work specifically on weak exchangers appears to be that reported by WIELAND AND BERG[10] and LEDERER[11] who have studied the migration of cations on carboxylic exchange papers.

EXPERIMENTAL

Preparation of cortical cells

The following enzymic digestion is essentially that of BLACKBURN[12]. Commercially scoured wool of 64s quality (60 g) was digested at 65° for 3 h in an aqueous solution (2 l), containing sodium bisulphite (20 g) and papain (20 g), which had been adjusted to pH 6.5 by addition of 1 *M* sodium hydroxide solution. Prolonged digestion led to an increased proportion of small particles which when incorporated into layers, markedly decreased the rate of development of chromatograms, particularly with aqueous solvents. After digestion of the fibres, cortical cells were recovered by filtration and then washed with distilled water (3 × 500 ml). The cells were then suspended in distilled water (300 ml) and the pH of the solution was adjusted to 3.0 by the careful addition of 6 *M* hydrochloric acid. The cells were heated in this suspension at 75° for 30 min, filtered, washed and stored in distilled water containing a little chloroform to inhibit growth of micro-organisms. The acidification and washing procedures were required to remove sodium bisulphite.

Chemical modification of cortical cells

Esterification. Oven-dried (100°, 1 h) cortical cells (9 g) were stirred in methanol (200 ml) containing concentrated hydrochloric acid (2 ml) and heated under reflux for 8 h. The cells were collected on a sintered glass filter and suspended in distilled water (100 ml). The pH of this slurry was adjusted to 6.5 by the addition of 1 *M* sodium hydroxide solution and checked from time to time during 0.5 h. The cells were then collected, rinsed with methanol and stored under methanol.

Deamination. Moist cortical cells (5 g) were suspended in 3.7 *M* sodium nitrate solution (165 ml) containing glacial acetic acid (35 ml) and stored in a lightly stoppered flask at room temperature for 24 h. The cells were then distinctly yellow. They were collected by filtration, washed with distilled water (4 × 300 ml), filtered and formed into layers immediately.

Preparation and properties of thin layers

Moist cortical cells (14 g) were suspended in 100 ml of an ethanol–water mixture by high speed homogenisation. For unmodified cells, an ethanol–water proportion of 1:1 was used whilst for esterified and deaminated cells it was 7:3. The composition of these mixtures had a considerable influence on the viscosity of the resulting slurry and this ultimately affected the ease of layer formation. After standing for

few minutes to allow bubbles to escape, the slurry was layered on to glass plates using the Desaga* apparatus set at 0.4 mm. The layers were allowed to dry at room temperature for at least 16 h. When dry, the layers were extremely robust and rigidly adhered to the glass plates. A micrograph of the surface of a cortical cell layer taken with a scanning electron microscope is shown in Fig. 1. It can be seen that many cortical cells have fragmented during digestion and that subsequent intertwining of filamentous structures in drying accounts for the excellent cohesion of the layers.

Keratin layers could be removed from glass surfaces and recovered intact after brief immersion (1–2 min) of the plate on which they were formed in hot water (70°). After drying, the separated keratin film had a papery texture and retained its characteristic strength and flexibility. Fig. 2 shows such a keratin film.

Pretreatment of layers

Each layer was wet out and allowed to stand for at least 4 h in the solution

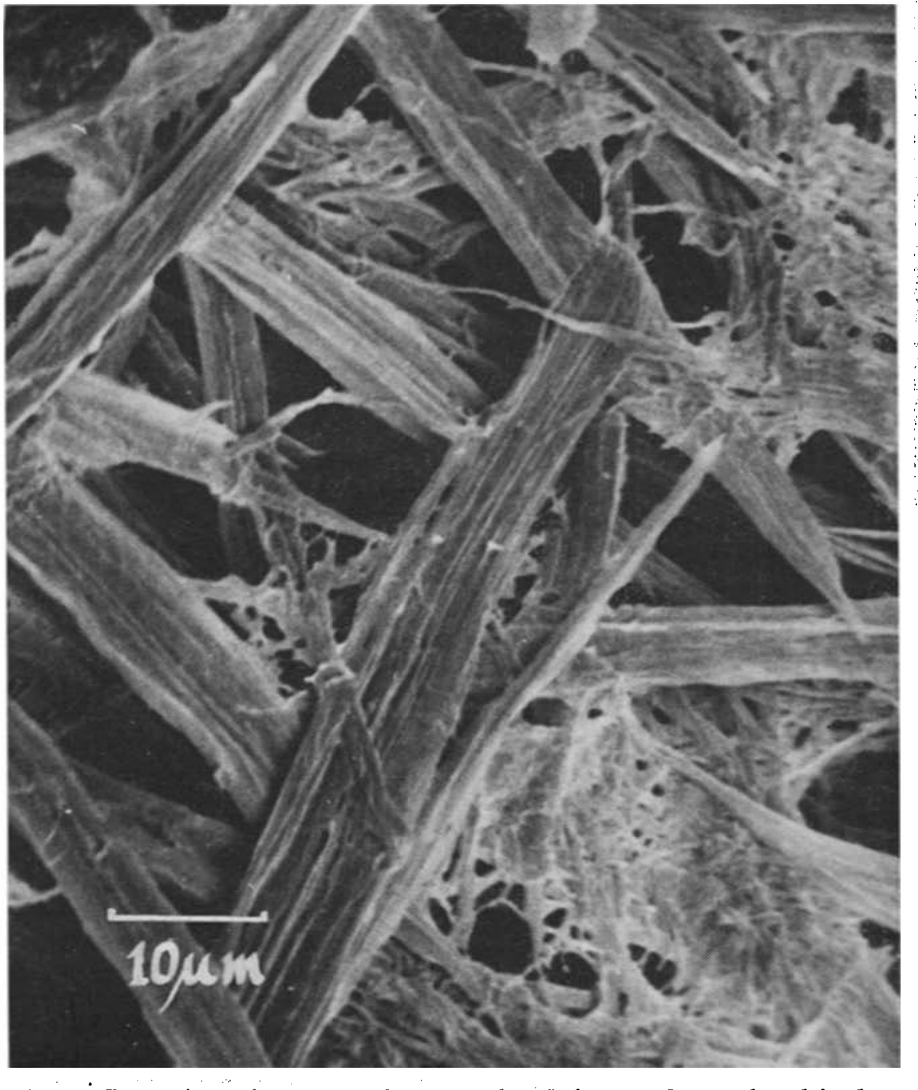

Fig. 1. Scanning electron micrograph of the surface of a thin layer of wool cortical cells.

* C. Desaga, G.m.b.H., Hauptstrasse 60, Heidelberg, G.F.R.

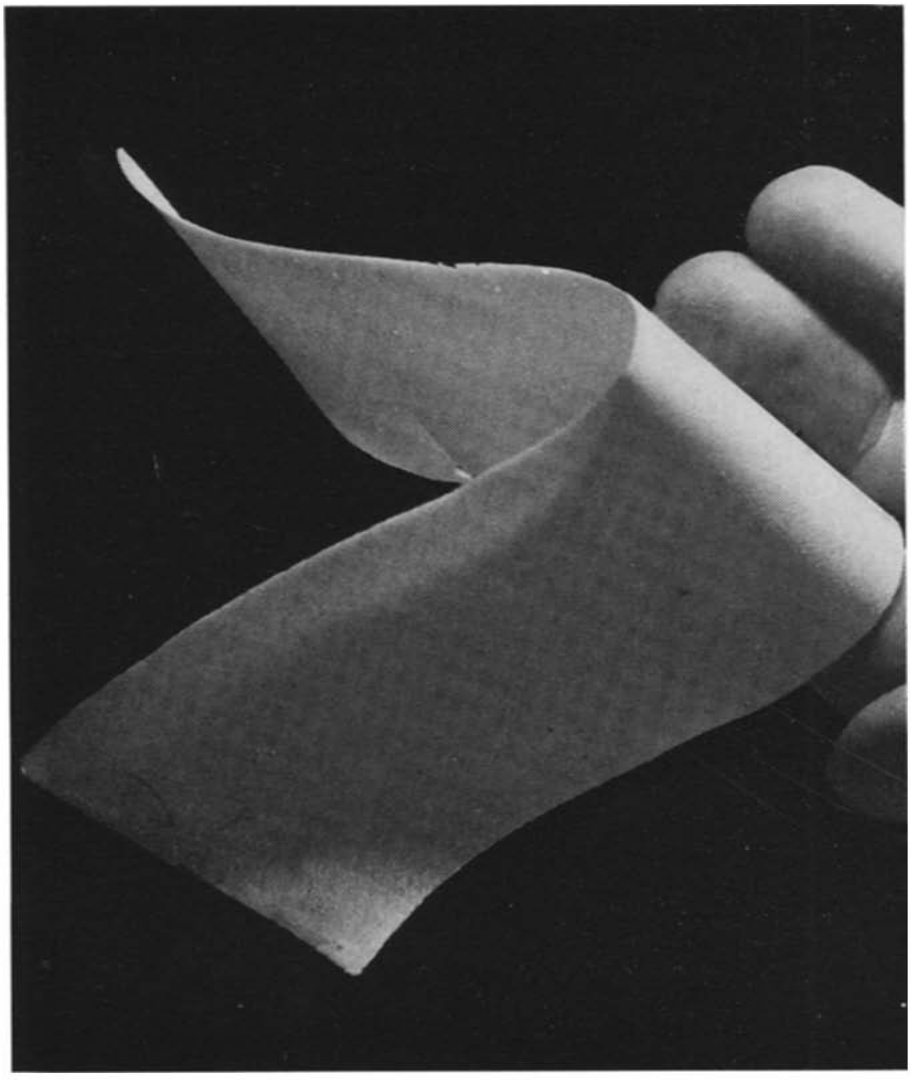

Fig. 2. A thin keratin layer after isolation from a glass plate.

(1000 ml) which was subsequently used for chromatographic elution. In this manner the pH of the keratin layer was equilibrated with that of the eluant. In order to minimise differences of pH of the cortical cells and the solutions, which may arise by the Donnan effect[13], the ionic strength of all the solutions used was at least 0.2. The internal cohesion of the layers prevented any disintegration during this soaking treatment. After equilibration, plates were rinsed by dipping once in distilled water and then allowed to drain and dry at room temperature. When dry, the layers were trimmed by removing a strip of cells around the edges of the plates and, as required, the layers were divided into a number of narrow segments simply by scoring channels in the layers with a narrow chisel. Substances could be chromatographed on individual segments of the layer. Layers were sufficiently robust for the starting points to be marked with a soft lead pencil.

Solutions of cations and anions

The concentration of salt in spotting solutions of the cations and anions was

20 mg/ml. Chlorides or nitrates of cations and sodium or potassium salts of anions were used. All chemicals were AR grade.

Development and visualisation of chromatograms

After spotting with the salt solution (1 μl), the chromatograms were developed in the ascending mode using normal techniques. After development, the liquid front was marked in pencil, the chromatograms were dried in air and the ions visualised by means of spray reagents. Not all reagents in common use are suitable for keratin layers. Those found successful are listed in Table I. Particular applications are given

TABLE I

SPRAY REAGENTS SUITABLE FOR USE ON KERATIN LAYERS

Reagent	*Composition*[a]
1	1 *M* Ammonia solution saturated with hydrogen sulphide
2	0.5% 8-Hydroxyquinoline in 60% aqueous ethanol. Layers were exposed to ammonia vapour after spraying and examined under UV light
3	0.2% Quinalizarine in ethanol
4	0.2% Aqueous aluminon
5	5% Pyrogallol in 5 *M* aqueous ammonia
6	0.1% Diphenylcarbazide in ethanol
7	0.4% Ammonium molybdate in 8% nitric acid followed by (8) after drying
8	1% Stannous chloride in 10% hydrochloric acid
9	1% Aqueous ferrous sulphate
10	1% Aqueous ferric nitrate
11	5% Potassium iodide in 2 *M* hydrochloric acid
12	Fresh aqueous solution containing 3% silver nitrate and 0.3% fluorescein. After spraying spots were developed by exposure to UV light

[a] All percentages are w/v.

in Tables II and III. Most ions were run singly on strips and it was possible to spray each strip individually by masking the rest of the plate. Substances requiring different visualising reagents could therefore be chromatographed on the same plate.

Unless otherwise stated, the ions migrated as discrete spots. The R_F values quoted in the tables are arithmetic means of at least three different determinations. In no case was the total variation in R_F greater than $\pm$ 4%.

RESULTS AND DISCUSSION

Cations

The R_F values of a number of cations obtained with phthalate buffers on normal and deaminated wool plates are shown in Table II. On unmodified keratin, all the metal ions were found to be more mobile at pH 4 than at pH 5. This is consistent with ion exchange of cations on carboxylate groups in the keratin. The pK_a of carboxyl groups in wool is of the order of 4[1] and it follows that at pH 4 about 50% of these groups will be protonated and unavailable for cation exchange. Therefore the proportion of cations bound at any time will be less than at pH 5 when all carboxyl groups are ionised. As R_F values are proportional to the ratio of concentrations

TABLE II

R_F VALUES OF CATIONS ON THIN KERATIN LAYERS

For spray reagents see Table I.
The solvent buffers were: (A) phthalate buffer, pH 4.0, ionic strength 0.2; (B) phthalate buffer, pH 5.0, ionic strength 0.2; (C) 0.2 *M* KNO_3 adjusted to pH 5.0 with nitric acid.

Cation	*Spray reagent*	*R_F values*			
		Unmodified keratin			*Deaminated keratin*
		A	*B*	*C*	*B*
Ag^+	1	× [a]	×	0.03T	—
Pb^{2+}	1	×	×	0.04T	—
Hg^{2+}	1	×	×	0.00	—
Fe^{3+}	1	×	×	0.00	—
Cu^{2+}	1	0.09T	0.04T [b]	0.05T	—
Cd^{2+}	1	0.59	0.49	0.22	0.16
Co^{2+}	1	0.72	0.63	0.47	0.22
Ni^{2+}	1	0.78	0.62	0.43	0.28
Mn^{2+}	2	0.84	0.74	0.49	0.32
Zn^{2+}	2	0.64	0.40	0.28	0.17
Cr^{3+}	2	0.47	0.30	0.20	—
Mg^{2+}	3	0.91	0.90	0.80	0.56
Al^{3+}	4	0.52	0.28	0.15	0.06T
Ca^{2+}	5	0.90	0.82	0.65	0.44
Sr^{2+}	5	0.89	0.82	0.63	0.44
Ba^{2+}	5	0.84	0.80	0.64	0.39
Sn^{4+}	6	0.05	0.04	0.02	—

[a] × Indicates that these cations precipitate in phthalate buffer.
[b] T Indicates tailing of spots.

of species free in solution to those bound on the exchanger[14] higher R_F values would be expected at pH 4 than at pH 5.

Several cations precipitated in the phthalate buffer, so R_F values were also measured in 0.2 *M* KNO_3 solution that had been adjusted to pH 5 by addition of dilute nitric acid. The R_F values of all cations that were mobile in phthalate buffer were found to be lower in the presence of potassium nitrate although their relative order of migration was not appreciably affected.

The migration of many of the metal ions was similar to that observed by LEDERER[11] on carboxymethyl cellulose paper (Whatman CM 50). This is illustrated in Fig. 3 in which the R_F values of the cations in phthalate buffer at pH 5.0 on unmodified keratin layers are plotted against LEDERER's values for the same metal ions in acetate buffer at pH 4.75 (0.5 *M* acetic acid, 0.5 *M* sodium acetate). A linear correlation between the R_F values on the two types of substrate is evident for all but Hg^{2+}, Ag^+, Cu^{2+} and the alkaline earth metals. Both Hg^{2+} and Ag^+ were strongly retained by keratin probably because they reacted with cystine disulphide bonds[15] which are plentiful in the cells*. Because of its tailing, the Cu^{2+} ion would also appear to react

* Native wool keratin has a sulphydryl content of about 35 μmoles SH (as cysteine) per g dry wool but this value can be increased to 500 μmoles SH per g by treatment with appropriate reducing agents. Measurements of the sulphydryl and disulphide content of the cells by the polarographic method of LEACH[17] indicated that they contained 80 μmoles of cysteine per g and 420 μmoles of cystine per g.

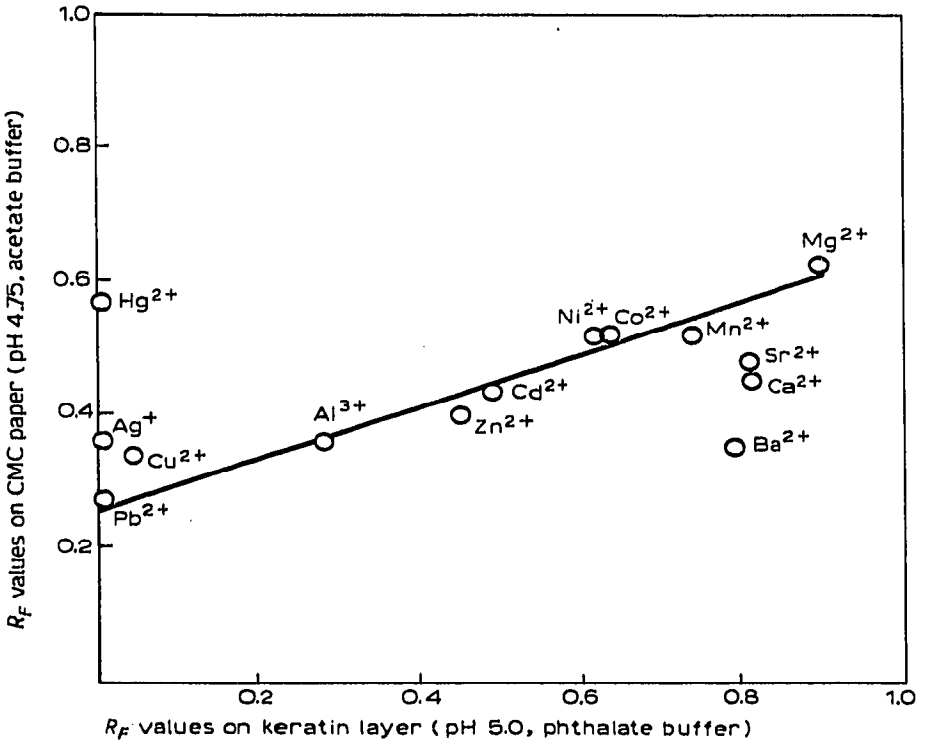

Fig. 3. Comparison of R_F values of metal ions on carboxymethyl cellulose paper in acetate buffer at pH 4.75 and on keratin in phthalate buffer at pH 5.0.

with wool to some extent. Ca^{2+}, Sr^{2+} and Ba^{2+} were not as strongly retained by keratin as by carboxymethyl cellulose and were not well resolved.

As can be seen from Table II, cations were more strongly retained by deaminated than by native keratin. The positively charged groups normally present in wool at values of pH less than 9 are removed by deamination leaving only carboxylate groups to interact with the cations.

It is important to bear in mind that because the buffer solutions contained concentrations of Na^+ and K^+ far in excess of the chromatographed ions the latter must have possessed greater affinity for keratin than either Na^+ or K^+; otherwise the chromatographed ions would have run up in the solvent front. In fact, DELMENICO AND PETERS[16] have shown that the sodium ion has little or no measurable affinity for wool and this is presumably true of the potassium ion.

Anions

The hydrogen phthalate anion was quite strongly bound on keratin layers because, of the anions examined in phthalate buffers, all but the dichromate anion (R_F 0.31) migrated close to the liquid front. The chromatography of anions was therefore studied only in the presence of chloride ion which has been shown to have little affinity for wool[16].

The R_F values of a number of anions on unmodified keratin at two different pH's are shown in Table III. The R_F values at pH 5.0 are in every case greater than at pH 2.5. At pH 5.0 the anions are adsorbed on the cationic protonated amino groups

TABLE III

R_F VALUES OF ANIONS ON THIN KERATIN LAYERS

For spray reagents see Table I.
The solvent buffers were: (A) HCl–KCl buffer, pH 2.5, ionic strength 0.2; (B) 0.2 *M* KCl adjusted to pH 5.0 with HCl.

Anion	*Spray reagent*	*R_F values*		
		Unmodified keratin		*Esterified keratin*
		A	*B*	*B*
$H_2PO_4^-$	7	0.72	0.85	0.78
$H_2P_2O_7$	7	0.30	0.81	0.45
$H_2PO_3^-$	7	0.65	0.90	0.76
$H_2PO_2^-$	7	0.65	0.88	0.73
SeO_3^{2-}	8	0.15T[a]	0.92	—
AsO_2^-	1	0.89	0 78	0.87
$Fe(CN)_6^{3-}$	9	0.05	1.0	0.07
$Fe(CN)_6^{4-}$	10	0.06	1.0	0.08
IO_3^-	11	0.15T	—	—
BrO_3^-	11	0.45	0.87	0.56
$Cr_2O_7^{2-}$	2	0.05	0.35	0.04
CNS^-	10	0.18	0.52	0.18
I^-	12	0.25	0.62	0.26
Br^-	12	0.39	0.78	0.41

[a] T Indicates tailing of spots.

but the concentration which can be bound is less than at pH 2.5 when the carboxylate groups are protonated and uncharged.

The R_F values of anions on unmodified wool at low pH are similar to those on esterified wool at pH 5.0 as shown in Table III. In both cases the carboxyl groups are uncharged and the keratin behaves exclusively as an anion exchanger.

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