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ELECTROPHORESIS OF THIOLS IN CELLULOSE GELS

I. GENERAL APPROACH

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SUMMARY

Cellulose gels have been produced by decomposition of viscose with ethylenediamine and potassium hydroxide in glass tubes. The rods obtained have been used for resolving thiols in acidic and neutral media in a xylene bath. The thiols are extracted from aqueous solution as tributyltin mercaptides followed by stripping with hydrochloric acid in glycerol, and a 0.2-ml sample is placed at the top of the gel which is in a vertical position. A preliminary separation of thiols into five groups based on the extraction properties is recommended. A method for reducing disulphides based on the use of tin(II) as the reducing agent and extraction of thiols as tributyltin mercaptides is suggested. The application of the complex of tetramercurated fluorescein with uric acid for the detection and fluorimetric determination of thiols is described.

INTRODUCTION

In our efforts to separate and identify thiols in biological materials, the analytical and preparative application of electrophoresis is expected to play an important role. The ionogenic thiols can be simply resolved by high-voltage paper electrophoresis[1] but the adaptation of the method to quantitative determinations was not successful.

Gel electrophoreses gives high resolution but the materials available are not suitable for thiols. Polyacrylamide gel contains acrylamide and additives that react with thiols. Agarose gels cannot be used for high-voltage electrophoresis which is required for thiols. It has been found, however, that the cellulose gel obtained by slow gelatinization of viscose (the solution of cellulose xanthate used for the production of cellulose fibres) in alkaline solution has excellent properties as a material for electrophoresis and is especially suitable for the resolution of thiols. The gel is chemically inert, mechanically strong and temperature resistant. In the course of electrophoresis it can be directly cooled in a toluene or xylene bath.

In order to explore the thiol composition in biological materials the following

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steps are required: (1) reduction of disulphides; (2) separation of thiols and preparation of the sample for resolution; (3) resolution into constituents; and (4) determination and identification of the resolved constituents. These steps are considered in this paper.

EXPERIMENTAL

Preparation of cellulose gels and electrophoresis

Cool 1000 ml of industrial viscose to 5°C and add, with mixing, 50 ml of 2 *M* potassium hydroxide solution and a cooled mixture composed of 100 ml of ethylenediamine and 100 ml of water. Mix to give a homogeneous solution and fill the mixture into glass tubes of length 250 mm and I.D. 12 mm. Within 24 h at room temperature the contents of the tubes solidify, forming cellulose gels. The cellulose xanthate is decomposed to form cellulose gel, sulphide and thiourea:

$$\text{Cel-O-CSS}^- + \text{C}_2\text{H}_8\text{N}_2 + \text{OH}^- = \text{Cel-OH} + \text{S}^{2-} + \text{C}_3\text{H}_6\text{S} + \text{H}_2\text{O}$$

Place the tubes in hot water to complete the decomposition, remove the gels from the tubes and wash them with water until no sulphide can be detected. Heat then the gels for 2 h at 90°C in 5% sodium sulphite solution, wash again with water, soak for 24 h at room temperature in 0.02 *M* EDTA-Na_2, wash with distilled water, soak for 24 h in 60% ethyleneglycol solution, remove and store in a closed vessel.

When the gels are equilibrated in the buffer solution and then applied directly for electrophoresis, the resolution is distorted by surface effects. The heat produced in the course of electrophoresis in the bulk of the gel is transferred from the surface to the xylene bath. Consequently, a temperature gradient and conductivity gradient will be formed, the speed of migration in the centre will be higher than on the surface and the migrating substances form cones. To overcome this phenomenon two buffers differing in conductivity are applied. The rods are first equilibrated for at least 24 h in a buffer containing 60% of ethyleneglycol and just before the electrophoresis are soaked for 5 min in buffer of the same pH but of higher conductivity in 5% ethylene glycol solution. Such treatment eliminates the surface effects because the temperature gradient and conductivity gradient cancel the effects of each other and the substances migrate in well defined parallel zones. The recommended compositions of the buffer solutions are given in the Table I. The alkaline buffers were not used because in alkaline solution thiols are readily oxidized by air.

The apparatus used for gel electrophoresis is shown in Fig. 1. In version (b) the electrode at the top is separated by a porous ceramic tube. It consists of a vessel containing buffer at the bottom and xylene in the upper layer. The liquids are cooled by a water cooler (not shown). The voltage is supplied by means of two platinum wire electrodes, one at the bottom and second at the top.

The procedure using version (a) is as follows. A rod of cellulose gel is placed tightly in a glass tube of I.D. 7 mm. The sample, consisting of thiols dissolved in glycerol, is applied on the top of the gel in the tube. The next upper layer in the tube forms buffer containing 60% of ethyleneglycol and the following upper layer forms buffer containing 5% of ethyleneglycol. The tube is fixed in the apparatus so that the gel must pass through the xylene layer and contact the solution at the bottom. The

TABLE I

COMPOSITION OF THE SOLUTIONS USED FOR THE PREPARATION OF CELLULOSE GELS AND IN ELECTROPHORESIS

Component	*pH 1.8*		*Component*	*pH 7.2*	
	1B2	*2B2*		*1B7*	*2B7*
Acetic acid	22.5 (v/v)	7.5 (v/v)	Triethanolamine	1.6 (w/v)	0.4 (w/v)
Formic acid	7.5 (v/v)	2.5 (v/v)	Boric acid	4.0 (w/v)	1.0 (w/v)
Ethylene glycol	60.0 (v/v)	5.0 (v/v)	EDTA-Na_2	0.05 (w/v)	0.05 (w/v)
Water	10 (v/v)	85 (v/v)	Ethylene glycol	60 (v/v)	5 (v/v)
			Water	To 100 ml	

complete arrangement of version (a) can be listed from the bottom as follows:

pH 1.8: cathode, solution 2B2, gel equilibrated with solution 1B2 and soaked for 5 min in solution 2B2, sample dissolved in glycerol, solution 1B2, solution 2B2, anode;

pH 7.2: anode, solution 2B7, gel equilibrated with solution 1B7 and soaked for

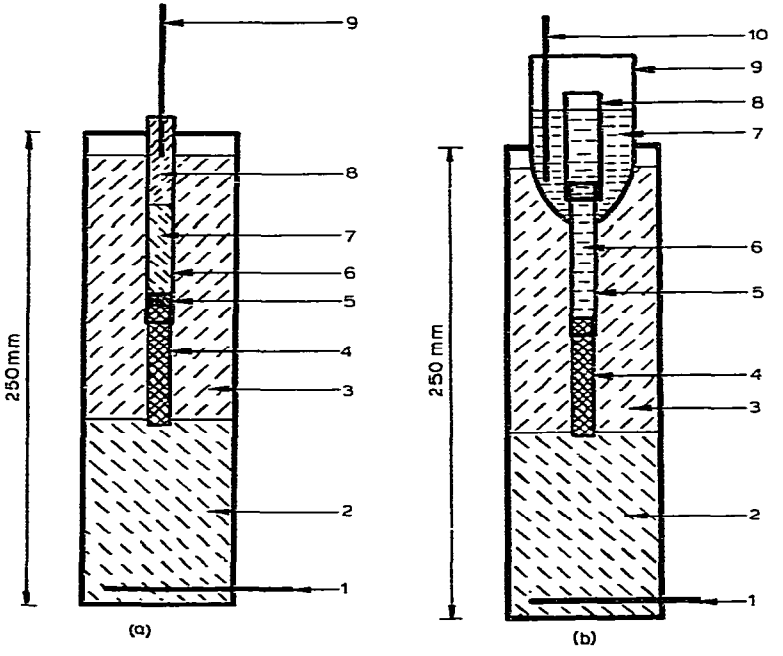

Fig. 1. The arrangement for electrophoresis in cellulose gels. Version (a): 1 and 9 = platinum-wire electrodes; 2 = solution 2B2 or 2B7; 3 = xylene; 4 = cellulose gel in the form of a rod of diameter 7 mm; 5 = sample dissolved in glycerol; 6 = glass tube of length 90 mm and 7 mm I.D.; 7 = solution 1B2 or 1B7; 8 = solution 2B2 or 2B7. Version (b): 1 = platinum cathode; 2 = solution 2B7; 3 = xylene; 4 = cellulose gel; 5 = glass tube of length 60 mm and 7 mm I.D. combined at the top with a glass vessel (9) of 30 mm I.D. and with a porous ceramic tube (8) of length 40 mm, O.D. 12 mm and I.D. 9 mm; 6 = solution 2B7; 7 = solution 1B7; 10 = platinum anode in the form of a ring.

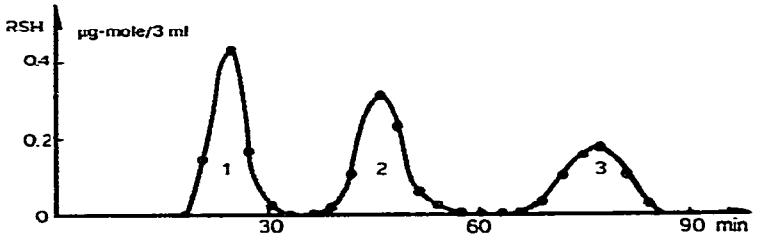

Fig. 2. Distribution curves at pH 1.8. Length of the gel, 50 mm. 1 = Cysteine methyl ester; 2 = cysteine; 3 = glutathione.

5 min in solution 2B7, sample dissolved in glycerol or 50% sucrose, solution 1B7, solution 2B7, cathode.

The current was always maintained at 12 mA, the corresponding voltage being about 100 V per 1 cm of the gel. When an electric current is applied the sample components will migrate downwards into the gel. The quantitative determination of the separated thiols can be effected either by cutting, pulverization and elution of the localized zones, or by removing the thiols migrating to the elution chamber. In the first method the gel containing the separated thiols is removed and either the thiols are localized directly by pressing the gel against a strip of Whatman paper followed by spraying with a suitable reagent or indirectly calculated from the position of the markers [the following are recommended: at pH 1.8, thiamine localized after spraying with potassium hexacyanoferrate(III)–sodium hydroxide reagent in UV light; and at pH 7.2, naphthionic acid localized directly in UV light], if the migration ratio of the thiols to the markers is known.

In the second method, which may be described as inverse electrophoresis, the gel is removed after 15 min and fixed in the same tube in the inverse position. At the same time the cathode and anode are exchanged. The thiols then migrate upwards but in the same direction within the gel and appear one after the other in the solution in the tube above the gel. At pH 1.8 version (a) is used and the chamber solution, 0.02 *M* orthophosphoric acid, is exchanged every 3 min, the volume being 3 ml. At pH 7.2 the first step is carried out according to version (a) but the second step must be carried out according to version (b) in order to separate the anode from the emerging thiols. Solution 2B7 is used for collecting the thiols and solution 1B7 as the anolyte. The same rate of exchange of 3 ml per 3 min was used.

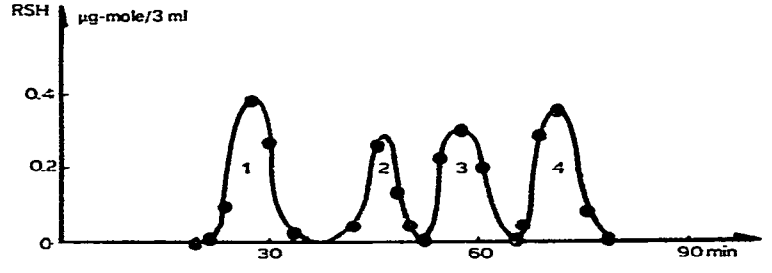

Fig. 3. Distribution curves at pH 7.2. Length of the gel, 59 mm. 1 = Thiomalic acid; 2 = N-acetylcysteine; 3 = captopril; 4 = glutathione.

Typical distribution curves obtained at pH 1.8 and 7.2 taking the 0.2-ml samples and using the method of inverse electrophoresis are demonstrated in Figs. 2 and 3. The time is calculated as the sum of steps 1 and 2. The recovery of thiols in acidic solution is nearly 100%, and about 90% in neutral solution.

Detection and determination of thiols

The titration of thiols with *o*-hydroxymercuribenzoic acid (HMB) in the presence of sodium or potassium hydroxide using dithizone as a visual indicator can be recommended for general use[2]. However, to obtain satisfactory results some conditions should be maintained, *viz.*, (1) the concentration of sodium hydroxide should be maintained between 0.05 and 0.20 *M* in excess with respect to the acid and amino salt content of the sample; (2) the amount of dithizone added should be kept to the minimum; and (3) the volume of the titrant required for complete conversion of the colour should not exceed 0.2 ml. The 3-ml fractions can be conveniently titrated with 10^{-4} *M* HMB, but even titration with $2 \cdot 10^{-5}$ *M* HMB in test-tubes was found to give satisfactory results.

The localization of thiols on the paper can be effected by spraying either with the dithiofluorescein–HMB complex or with tetramercurated fluorescein (TMF)[1]. A very sensitive reagent for the detection and determination of thiols has been prepared from TMF and uric acid. The complex between TMF and uric acid does not fluoresce, but the complexes of TMF with mercaptoacids show a strong fluorescence[3]. As the thiols displace the uric acid from its complex with TMF to form fluorescent complexes, the appearance of fluorescence indicates the presence of thiols that form fluorescent complexes with TMF.

Procedure. Prepare solution A by dissolving of 17 mg of TMF tetraacetate in 20 ml of 0.1 *M* potassium hydroxide solution and diluting to 100 ml. Prepare solution B by dissolving 17 mg of uric acid in 100 ml of 0.02 *M* lithium carbonate solution.

For detection of thiols on the paper, mix 2 ml of solution A, 2 ml of solution B and 6 ml of 2% diethanolamine solution. Spray the paper with the reagent and observe it under filtered UV light. The thiols appear as yellow spots on a dark background. The limit of detection for mercaptoacids is 1 pmole/cm^2.

The fluorimetric determination of thiols in the 3-ml fractions in solution 2B7 can be carried out as follows. Prepare the reagent by mixing 2 ml of solution A and 1 ml of solution B followed by dilution to 50 ml with 0.05 *M* borax solution. The reagent should be used within 1 day. Add 0.5 ml of the reagent to every 3-ml fraction of solution 2B7 collected during inverse electrophoresis. At the same time prepare as reference a solution containing 0.2 μmole of cysteine and 0.5 ml of the reagent. Prepare also a blank from 3 ml of solution 2B7 and 0.5 ml of the reagent. Keep the samples in the dark for 20 min and then measure the fluorescence with an exciting wavelength of 515 nm. The blank is taken as zero and the reference with cysteine as 100.

Some curves obtained in Zeis Jena Spekol spectrofluorimeter using test-tubes of 16 mm diameter are shown in Fig. 4. It can be seen that above 2 nmole of thiol a linear relationship is established. The fluorimetric method is very sensitive and may be expected to be adaptable for monitoring thiols. However, for each thiol a calibration graph must be constructed.

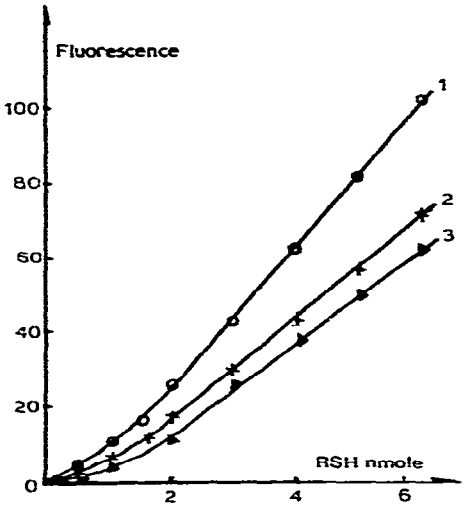

Fig. 4. Fluorescence at 515 nm of exciting wave as function of the thiol content in 3.5 ml of solution containing TMF and uric acid. 1 = Glutathione; 2 = N-acetylcysteine; 3 = captopril.

Reduction of disulphides

The experimental evaluation of different methods described in the literature[4] has shown that the electrolytic reduction on a lead cathode in the presence of sulphuric acid may be recommended for the reduction of disulphides in diluted aqueous solutions, *e.g.*, in urine. The high concentration of proteins, however may, interfere with the reduction because they deposit on the cathode, slowing the reduction and producing foam. For such samples another procedure, described below, may be recommended. It is based on the use of tin(II) ions, which are powerful reducing agents and have already been suggested for the reduction of inorganic polysulphides[5]. The course of the reduction of disulphides can be followed by titration of the produced thiols with *o*-hydroxymercuribenzoic acid (HMB) and when the reduction is completed the thiols can be separated by extraction as tributyltin mercaptides. The excess of tin(II) has to be masked with EDTA.

Preparation of tin(II) chloride solution. Dissolve while heating 5 g of tin(II) chloride ($SnCl_2 \cdot 2H_2O$) in 5 ml of 40 % hydrobromic acid and dilute to 50 ml. Store in a brown flask under octane at 5°C.

Procedure. Add to a test tube-containing 2–5 ml of the sample 0.2 ml of the tin(II) solution and 0.8 ml of 2 *M* potassium hydroxide solution. Place the test-tube in boiling water or keep it at room temperature, depending on the sample, cool quickly, add 1 ml of hexane to prevent air oxidation, add 1 ml of 0.1 *M* EDTA-Na_2 solution, 0.6 ml of 1 *M* orthophosphoric acid, to give a pH of about 9, and titrate with HMB solution in the presence of dithiofluorescein as indicator to the complete disappearance of the blue colour. Using a blank sample, determine the consumption of HMB for the dithiofluorescein added and subtract from the result (about 0.3 ml of 10^{-4} *M* HMB).

Some results obtained by the procedure are given in Table II.

TABLE II

REDUCTION OF DISULPHIDES BY TIN(II) FOLLOWED BY TITRATION OF THE THIOLS PRODUCED WITH 10^{-4} *M* HMB

Disulphide	*Amount taken (μmole)*	*Amount found (μmole)*	*Time (min)*	*Temperature (°C)*	*Mean recovery (%)*
Dithiodiacetic acid	0.205	0.205,0.200,0.205	10	20	99
	0.410	0.405,0.405,0.415	10	20	99
2,2'-Dithiodipropionic acid	0.185	0.180,0.190,0.185	10	100	100
	0.370	0.365,0.380,0.365	10	100	99
Oxidized captopril	0.215	0.215,0.215,0.212	10	100	99
	0.430	0.415,0.410,0.415	10	100	96
Cystine	0.255	0.215,0.220,0.235	5	100	87
	0.510	0.490,0.495,0.480	5	100	96
Homocystine	0.224	0.230,0.227,0.220	5	100	100
	0.448	0.450,0.430,0.445	5	100	99

Classification of thiols and preparation of samples for electrophoresis

The classification of thiols presented is based on their ability to be extracted with organic solvents. The extraction of thiols as tributyltin mercaptides has been described elsewhere[6,7]. As demonstrated in Table III, the thiols are divided into five groups on the basis of the extraction with hexane, a solution of TBT in hexane, a solution of TBT in butanol and stripping with dilute hydrochloric acid.

The thiols in group 0 do not have hydrophilic functions and can be extracted with organic solvents without TBT at pH 7. Group 1 includes higher mercaptoacids such as dihydrolipoic acid. They can be extracted with organic solvents from acidic media, but from neutral media only as tributyltin mercaptides. The thiols in group 2 can be extracted at pH 6 (acidic thiols) or at pH 9 (alkaline thiols) with hexane only as tributyltin mercaptides, but are stripped completely with hydrochloric acid, differing in this respect from the thiols in group 1. The thiols in group 2 include cysteamine, cysteine methyl ester, thioglycollic acid, thiolactic acid, thiomalic acid, mercaptolactic

TABLE III

CLASSIFICATION OF THIOLS

Group	*Extraction at pH 6–9 with*			*Stripping with 0.2 M HCl*
	Hexane	*TBT in hexane*	*TBT in butanol*	
0	Strong	Strong	Strong	None
1	None	Strong	Strong	Moderate
2	None	Strong	Strong	Strong
3	None	Weak	Moderate	Strong
4	None	None	Weak or none	Strong

TABLE IV

SCHEME FOR SEPARATION OF THIOLS INTO GROUPS BY EXTRACTION AND STRIPPING

Volume of sample, 100 ml.

Extraction with 3 × 5 ml of hexane
Organic phase: group 0
Sample: extraction with 3 × 3 ml of 0.2 *M* TBT in hexane
Organic phase: stripping with 3 × 4 ml of 0.2 *M* HCl
Organic phase: group 1
Aqueous phase: washing with 3 × 10 ml of hexane
Organic phase: group 1
Aqueous phase: group 2
Sample: extraction with 3 × 10 ml of 0.3 *M* TBT in butanol
Organic phase: group 3
Sample: group 4

acid, captopril and other thiols with moderate hydrophilic properties. The thiols in group 3 have strong hydrophilic properties, but can still be quantitatively extracted as tributyltin mercaptides with butanol. Group 3 includes cysteine, homocysteine, penicillamine and 2-mercaptopropanosulphonic acid. The thiols in group 4 cannot be extracted with a solution of TBT in butanol. Their tributyltin mercaptides are only slightly soluble in butanol and either remain in the aqueous phase or form precipitates in the organic phase. The precipitate is probably produced from peptides and proteins containing many mercapto functions. Glutathione should be classified between groups 3 and 4.

The scheme for the separation of thiols into the five groups without taking into account the precipitation is demonstrated in Table IV.

The preparation of the samples for electrophoresis is a crucial aspect of the analysis. The concentrated sample can simply be mixed 1:1 with glycerol or at pH 7 with sucrose and used for the separation. If the thiols are first separated by extraction they have to be stripped with a small volume, say 0.5 ml, of glycerol and 0.2 ml is taken for resolution. The stripping is performed with a solution of hycrochloric acid in glycerol but excess of acid must be avoided. It is suggested that phenol red can be used as an indicator; between pH 1 and 2 the colour changes from yellow to rose. The procedures for groups 2 and 3 may be outlined as follows.

Group 2. Extract a 5–20-ml sample with 2 ml 0.03–0.2 *M* TBT in hexane, wash the extract with water and 5% sodium sulphite solution containing 1% sodium bisulphite (pH 7) and transfer into a test-tube of 7 mm diameter with a conical-shaped bottom. Add 0.01 ml of phenol red solution (1 mg/ml in 50% ethanol) and titrate with 0.2–0.5 *M* hydrochloric acid in glycerol, shaking strongly after each drop until the colour of the indicator changes. Remove the hexane by decantation and wash with shaking three times the glycerol with 1 ml of pure hexane to remove TBT chloride and the thiols of group 1. Take 0.2 ml of the glycerol layer for electrophoresis at pH 1.8, but for electrophoresis at pH 7.2 first neutralize the sample by shaking with 1 ml of a 0.5 *M* solution of dibutylamine in hexane.

Group 3. Saturate a 10-ml sample after extraction of group 2 thiols with butanol and extract with 2 ml of 0.3 *M* TBT in butanol at pH 7. Wash the extract with water saturated with butanol and titrate with 0.5 *M* hydrochloric acid in glycerol

using phenol red as indicator. Add 2 ml of hexane, remove the upper phase by decantation and wash the glycerol phase three times with 1 ml of hexane. Use the sample for electrophoresis at pH 1.8 and 7.2 as for group 2.

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