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ISOELECTRIC FOCUSING OF LENS CRYSTALLINS IN THIN-LAYER POLYACRYLAMIDE GELS

A METHOD FOR DETECTION OF SOLUBLE PROTEINS IN EYE LENS EXTRACTS

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SUMMARY

The high resolving capacity of isoelectric focusing in thin layers of polyacrylamide gel was utilized for the separation of lens crystallins from eye lens tissue. Isoelectric points of the crystallins were determined. A micro-scale method for separation of the same proteins is also described.

INTRODUCTION

In protein chemistry, isoelectric focusing is a very powerful method for the separation of proteins. The determination of the isoelectric points of the separated protein components provides a basis for the physico-chemical characterization of these components. SVENSSON[1] and VESTERBERG AND SVENSSON[2] developed the technique of isoelectric focusing in sucrose density gradients with "Ampholine" carrier ampholytes (LKB-Produkter, A.B., Stockholm, Sweden). Much work has already been done on isoelectric focusing in sucrose gradients. A number of reports have also been published on isoelectric focusing in acrylamide gel. FAWCETT[3], DALE AND LATNER[4], and WRIGLEY[5] used the method of disc-isoelectric focusing, but only a few reports are available on thin-layer polyacrylamide gel-isoelectric focusing. LEABACK AND RUTTER[6], and AWDEH *et al.*[7] used this thin-layer technique which shows the advantage of a direct comparison between the samples applied, as they are submitted to the same conditions on one gel slab. Moreover, the resolving capacity in thin-layer polyacrylamide gel is possibly higher than that in the disc method. HUMPHRYES[8] used the same thin-layer technique, and measured the pH gradient on the slab by cutting out and subsequently eluting the discs taken with a cork borer along the length of the slab.

The method described in this paper permits an exact comparison of the samples applied, and an accurate determination of the isoelectric points of the proteins separated, by direct measurement of the pH gradient with the aid of a surface glass electrode. Our isoelectric focusing technique was carried out in the same way as described by AWDEH *et al.*[7]. More details about the easy application of the method

are given, and also a convenient microtechnique is described. This micro scale method makes it possible to identify 0.1 mg of protein in the sample.

MATERIALS AND METHODS

Lens proteins

Normal human lenses were obtained from the dissection room of our department, mostly within 6 h after death. Cataractous human lenses were collected after cataract extraction in the University Eye Clinic, and stored at −20° until needed.

Chicken heads and adult cow eyes were transported from the slaughter house under ice. Lenses were carefully dissected free from the lens capsule and the surrounding tissue and homogenized in distilled water. Separation of lens cortex and nucleus was made by placing lenses in distilled water and by gently stirring for 2 h at 4°. After centrifugation at 49,500 × g for 30 min in a Sorvall refrigerated centrifuge, the supernatant was carefully removed and lyophilized.

Preparation of the acrylamide gel

A 5% (w/v) polyacrylamide gel containing 2% (w/v) "Ampholine" carrier ampholytes (LKB-Produkter, A.B., Stockholm, Sweden) was made. Stock solutions for preparing the gel were as follows:

(*A*) *Catalyst solution*: 1 ml N,N,N',N'-tetramethyl-1,2-diaminoethane (BDH Chemicals, Ltd., Poole, Great Britain; No. 30385), distilled water to 150 ml.

(*B*) *Acrylamide solution*: 100 g acrylamide (BDH Chemicals, Ltd., No. 27045), 2.7 g N,N'-methylenebisacrylamide (BDH Chemicals, Ltd., No. 29227), distilled water to 400 ml.

(*C*) *Photoactivation solution*: 2 mg riboflavin (E. Merck, A.G., Darmstadt, G.F.R., No. 7609), distilled water to 100 ml.

When kept at 4° in the dark, these solutions could be stored for at least one month.

Gel solution was prepared by mixing 7 ml of solution A, 14 ml of B, 18.5 ml of C and 3.3 ml of "Ampholine" carrier ampholytes, purchased as 40% solution, and covering the desired pH range. Water was added to 65 ml. The gel solution was prepared immediately before use. The "Ampholine" carrier ampholytes applied covered a pH range of 3–10, and consisted of 1.1 ml Ampholine pH 3–5 (No. 8151), 1.1 ml Ampholine pH 5–7 (No. 8153) and 1.1 ml Ampholine pH 7–10 (No. 8144). The Ampholine pH 3–10 (No. 8141) is also applicable, but gave a slightly lower resolution effect.

A gel mould was prepared with two glass plates (25 × 17.5 cm), clamped together in a vertical position and separated by a piece of flexible tubing of about 1.5 mm I.D., to enclose the gel solution. One of the glass plates had been siliconized chemically with dichlorodimethylsilane (b.p. 31°) as described below, and marked. Using a syringe, the gel mixture (58–65 ml) was carefully poured between the glass plates. Any air bubbles should be avoided. The solution was polymerized by photoactivation for 1–2 h at 20° using two 20 W fluorescent daylight TL tubes (Philips No. 55) placed vertically about 5–10 cm from the gel. After polymerization, the clamps and the tubing were removed and the siliconized glass plate was lifted off with a knife, allowing air bubbles to form between this plate and the gel while lifting. The

polymerized gel adheres firmly to the other glass plate. The gel prepared in this way may be used immediately or stored at 4° overnight until needed.

Coating by siliconizing glass plates

The glass plates to be used were siliconized by placing them in a well-closed box, with care that they did not touch each other. At the bottom of this box, a petri dish was filled with 5 ml of dichlorodimethylsilane (E. Merck, A.G., Darmstadt, G.F.R., No. 3452) and the plates were left for fourteen days in a fume cupboard. After this time, the lid was removed and the glass plates were rinsed well under the tap. The coating lasts indefinitely, and facilitates the easy removal of the treated glass plate from the gel. An alternative method, which is more convenient, is to siliconize the glass plate with a 2 % solution of dichlorodimethylsilane in carbon tetrachloride (Repelcote: Hopkins and Williams, Ltd., Chadwell Heath, Great Britain, No. MS 2217). The plate should be washed out with this solution, making sure that the glass is fully wetted, and the carbon tetrachloride allowed to evaporate.

Application of the samples

The protein samples were dissolved in distilled water to make a 2 % solution. Pieces of Whatman No. 3MM filter paper, 1 × 1 cm, were placed on the gel 15 mm from the anode, 1 cm apart at one of the short edges of the gel slab to become the anodic side. In the present experiment, 50 μl of the sample (corresponding to 1 mg of lyophilized lens extract) were applied to soak these filter-paper squares. It appeared that with lens proteins, the samples should be applied on the anodic side to avoid the negatively charged α-crystallins to precipitate[9] all over the gel, which would be the case when the sample was applied on the cathodic side. With anodic sample application, no artifacts[10] were observed. No more then six samples were run on a slab simultaneously.

Isoelectric focusing procedure

The isoelectric focusing was performed in an electrophoresis apparatus (MBI, Ltd., Ashford, Great Britain, Tank No. 260) on a Perspex stand, available as a spare part. Before placing the gel on the electrodes, the anode and cathode were moistened with 5 % (v/v) phosphoric acid and 5 % (v/v) ethylenediamine solution, respectively. The glass plate was inverted with the gel facing downwards and resting in a horizontal plane over two stainless steel electrodes placed 20 cm apart. Isoelectric focusing was performed at a constant current of 4 mA for 24 h at 4°. During this time, the voltage applied began at 45 V and was increased up to 250 V. The voltage applied should not exceed 250 V in order to prevent an electro-osmotic flow connected with shifting of the gradient[11]. At the end of the run, the current was actually less than 2 mA per gel. The gradient was established, when clearly visible refractile lines appeared in the gel, mainly at the anodic side. These lines should be strictly parallel to the electrodes. It is absolutely necessary that the steel or carbon rods are perfectly straight to obtain an even pH gradient. When the sample contains any salt, the gradient is distorted[12] and the protein bands are not arranged uniformly parallel to the electrodes.

When the voltage applied was too high, or when the time taken for the run was excessive, an electro-osmotic flow of water mainly to the cathode tended to decrease the thickness of the gel just half-way between the electrodes. Focusing times longer

than 24 h resulted in a compression and a cathodic migration of the pH gradient and the focused bands[11].

Determination of the pH gradient

Immediately after completion of the run, the pH gradient was determined at 4° with the aid of a Radiometer pH-meter. After establishing the contact between the flat circular underside of the calomel electrode (Radiometer type K 401) and the gel, measurements were taken with a flat membrane glass electrode, 17 mm in diameter (Radiometer type G 242C), at points 20 mm apart along the gel, at the same time marking positions with a 0.5 mm diameter punch. The pH gradient could also be conveniently determined with a combined micro-surface electrode, 6 mm in diameter (Ingold, A.G., Zürich, Switzerland, type LoT 403-30-M 8).

Removal of the "Ampholine" carrier ampholytes

The gel slab was washed 6 or 7 times with trichloroacetic acid solutions of gradually decreasing concentration, starting with 10 % and ending with 3 %. Each washing period lasted for at least 3 h. Before staining, the trichloroacetic acid was washed away repeatedly with methanol–acetic acid–water (45:9:46) until the pH of the gel was about 4.0, to avoid precipitation of the dye at low pH.

Protein staining

Staining was carried out in a 0.05 % solution of Coomassie Brilliant Blue R-250 dye (Serva, Heidelberg, G.F.R., No. 17525) in methanol–acetic acid–water (45:9:46) for 2 h. After staining, the gel slab was washed in the same solvent until the background was clear. After this treatment, the gel slab had shrunk considerably owing to water withdrawal. The gel was then placed in acetic acid–water (9:91) to swell it again and to facilitate its handling. Before taking photographs of the stained polyacrylamide gel, lead pellets were placed in the holes punched in the gel for the pH measurements. The pI values of the protein bands were obtained by referring the pH measurements (Table I) and the stained zones to a fixed reference point (Fig. 1). The pH gradient curve obtained (Fig. 2) was normally only slightly concave, and the

TABLE I

DETERMINATION OF THE pH GRADIENT CURVE

Measurements taken with a Radiometer surface glass electrode.

Beside A	*Between C and D*	*Beside E*	*Mean*
2.60	2.65	2.78	2.68
4.00	4.10	4.15	4.08
4.45	4.49	4.52	4.49
4.97	5.00	5.02	4.99
5.40	5.43	5.45	5.43
5.83	5.84	5.85	5.84
6.43	6.44	6.44	6.44
7.02	6.98	6.96	6.99
7.90	7.86	7.88	7.88
8.42	8.46	8.40	8.40
8.90	8.80	8.82	8.84

distance representing one pH unit tended to decrease in the cathodic direction (*cf.* the scale on Fig. 1). This results in a better separation ability on the anodic side.

Photography of results

The stained gels were photographed on Kodak Panatomic X film using transmitted light and a good diffusing screen uniformly illuminated. A steep red filter with absorption spectrum between 500 and 600 nm enhanced the contrast.

Conservation of the gel

A polyacrylamide gel is very difficult to handle and cannot be kept without

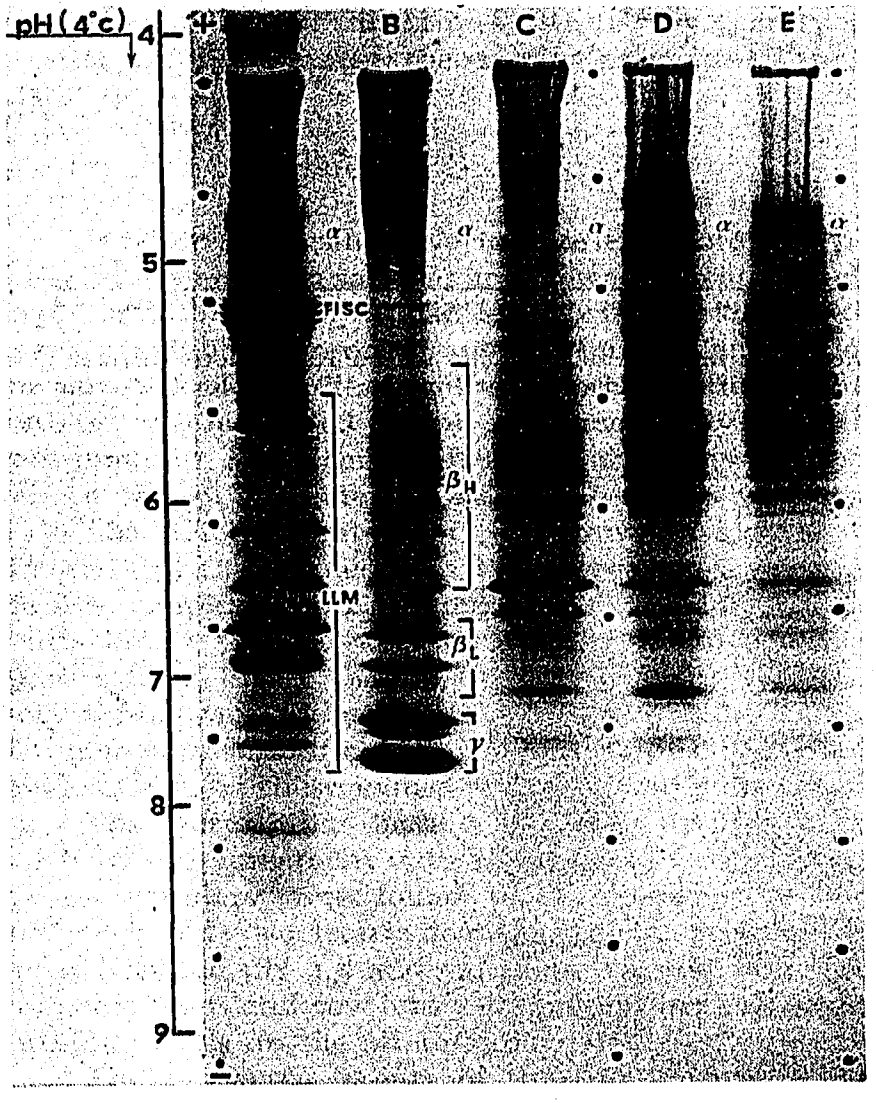

Fig. 1. Isoelectric focusing pattern of (A) total chick lens crystallins, (B) cow lens cortex crystallins, (C) human lens cortex crystallins (cataractous), (D) human lens cortex crystallins (normal), and (E) human lens nucleus crystallins (normal) in 5% polyacrylamide gel, 2% "Ampholine" carrier ampholytes, pH range 3–10 (see text). The samples A–E amounted to 1 mg of protein. The pH was measured with a flat-membrane glass electrode on the spaces between adjacent marks (see text). α = α-crystallins; FISC = First Important Soluble Crystallin; LLM = "Long Line Material"; β_H = β-crystallins of high molecular weight; β_L = β-crystallins of low molecular weight; γ = γ-crystallins.

special treatment. The stained gels were preserved by simply covering them with a layer of 7 % gelatin, white extra-pure (E. Merck, A.G., Darmstadt, G.F.R., No. 4072) and 5 % glycerin, and were dried for some days, according to the method described

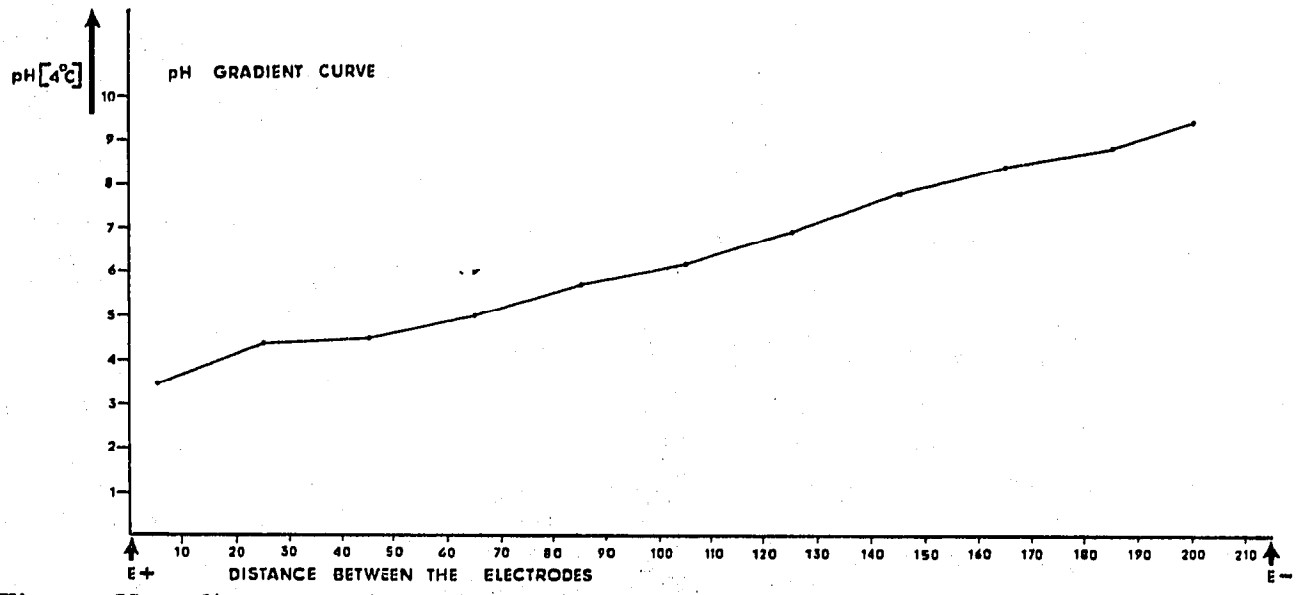

Fig. 2. pH gradient curve determined from measurements taken with a Radiometer surface glass electrode from a 5 % polyacrylamide gel after isoelectric focusing in "Ampholine" carrier ampholytes, pH range 3–10 (see text).

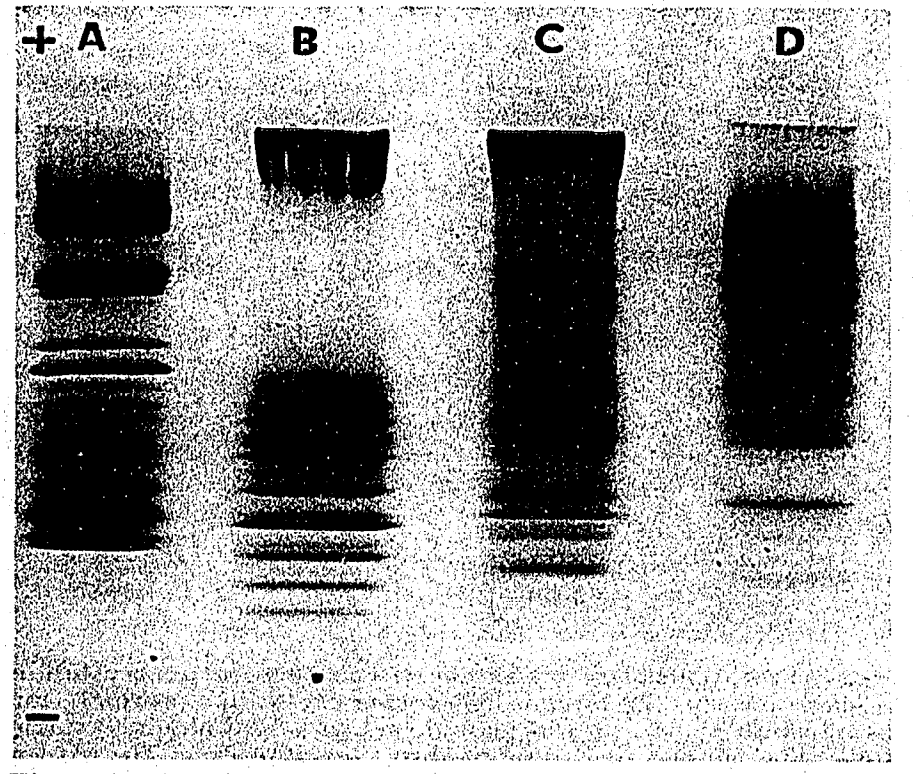

Fig. 3. Isoelectric focusing pattern of (A) total chick lens crystallins, (B) cow lens cortex crystallins, (C) human lens cortex crystallins (cataractous), and (D) total human lens crystallins (normal) in 5 % polyacrylamide gel, 2 % "Ampholine" carrier ampholytes, pH range 3–10 (see text). Size of the gel 9 × 9 cm. The samples amounted to 0.2 mg of protein.

by DANGERFIELD AND FAULKNER[13] for starch gels. The gel preserved in this way can be kept for a long time as a hard layer on the glass plate.

Micro method

The isoelectric focusing procedure described is also applicable on a micro scale. The gel was moulded between two lantern slides of 8.2 × 8.2 cm (Ilford Cover Glass), or on glass sheets of 9 × 9 cm. The amount of protein for every sample was 10 μl of a 2% solution, corresponding to 0.2 mg, on a 10 × 5 mm sample paper. The distance between the electrodes was 8 cm. The current was 2 mA per gel and the voltage applied was 40–70 V for 16 h at 4°, followed by 350 V for 15 min to sharpen the pH boundaries, resulting in sharp bands (Fig. 3). These gels of smaller size need only to be washed out three times with 10 %, 5 % and 3 % trichloroacetic acid solution, respectively. For the rest, the staining procedure is similar to that for the larger scale gels.

RESULTS AND DISCUSSION

Isoelectric focusing of vertebrate lens proteins in polyacrylamide gel is illustrated in Fig. 1. Five protein samples are shown: (A) total chick lens crystallins; (B) cow lens cortex crystallins; (C) human lens cortex crystallins (cataractous); (D) human lens cortex crystallins (normal); and (E) human lens nucleus crystallins (normal). After the run, the pH gradient in the gel could be seen to be perfectly straight. The pH gradient was measured on the gel immediately after the run and pH measurements were made at 4° on three different tracks between the electrodes (Fig. 1); the data were in perfect agreement (Table I).

The pH gradient curve was drawn using the mean values of Table I. The pI values of the stained protein zones were estimated and the isoelectric points of the five samples A to E are listed in Table II. The α-crystallins had their isoelectric points below 5.1 and were all determined to be near 4.80–4.85 and 4.95–5.00. It appears from Fig. 1 that in the soluble proteins of the human lens three different α-crystallins were present, with pI values of 4.85, 4.95 and 5.03. The isoelectric points of the components of chick lens α-crystallins were determined as 4.85 and 5.00. For cow lens cortex, these values were 4.80 and 5.00.

The α-crystallins have a tendency to precipitate[9] at their isoelectric points, which was in agreement with the results of column isofocusing experiments[14] where the α-crystallins were found to precipitate. This precipitation also occurred in polyacrylamide isoelectric focusing[15]. The precipitated α-crystallin appeared as a smear between pH 4.5 and 5.0 (Figs. 1A and 1B); more components of α-crystallins from chick lens and cow lens were present in the α-crystallin region of Fig. 1 than are actually visible on the photograph.

The isoelectric points of the components of chick FISC[15] (First Important Soluble Crystallin[16]) are listed in Table II. The chick FISC consisted of seven components, which showed immunologically a reaction of identity; details have been published earlier[15]. The chick FISC showed heterogeneity of a presumably homogeneous protein fraction[17]. The chick lens proteins from pI 5.68 to 7.58 have often been referred to as the "Long Line Material"[18]. As appears from other studies in progress, the pI values of the crystallins of cow lens cortex, from 5.70 to 6.60, are referred to as

β-crystallins of high molecular weight, and from 6.70 to 6.90 as β-crystallins of low molecular weight. From pI 7.00 to 8.10, γ-crystallins are present.

The human soluble crystallins from the cortex of the lens showed no qualitative

TABLE II

ISOELECTRIC POINTS OF LENS CRYSTALLINS

A, α-crystallins; B, FISC; C, "long-line material"; D, β-crystallins of high molecular weight; E, β-crystallins of low molecular weight; F, γ crystallins.

Total chick lens crystallins, normal	*Cow lens cortex crystallins, normal*	*Human lens cortex crystallins, cataractous*	*Human lens cortex crystallins, normal*	*Human lens nucleus crystallins, normal*
4.85 } A	4.80 } A	4.85 } A	4.85 } A	4.88 } A
5.00 }	5.00 }	4.95 }	4.93 }	4.95 }
		5.04 }	5.03 }	5.03 }
		5.11	5.10	5.08
5.20			5.20	5.21
5.22				
5.25	5.25		5.26	5.25
5.28 } B		5.28		
5.30		5.30		
5.32				
5.36		5.38	5.35	5.35
		5.40	5.40	5.45
		5.50	5.53	5.53
5.58		5.60	5.58	5.58
		5.65	5.63	5.63
5.68	5.70	5.69	5.70	5.68
5.70	5.72	5.73	5.75	5.73
5.78	5.80	5.78	5.79	5.75
5.80	5.82	5.83	5.80	5.80
5.82	5.85	5.88	5.85	5.85
5.90	5.90	5.95	5.93	5.93
6.00	6.00 } D			
6.05		6.06	6.00	6.00
6.10	6.10	6.13	6.08	6.13
6.20	6.16	6.18	6.18	6.17
	6.25		6.25	6.21
6.30 } C			6.35	6.28
6.40	6.40	6.43	6.48	6.50
	6.45			
		6.53	6.58	6.58
6.65	6.60			
	6.70	6.68		6.63
	} E	6.79		6.79
6.82			6.85	
6.90	6.92	6.93		
		7.03		
	7.10			7.15
		7.21	7.21	7.25
7.28	7.30	7.33	7.33	
	7.42 } F			
7.48			7.55	7.53
7.58		7.60		7.60
	7.68			
8.10	8.10			
8.20				
8.38				

difference between the normal proteins (Figs. 1D and 3D) and the cataractous proteins (Figs. 1C and 3C). This may be an indication that the cause of the cataract is not to be found in the difference between normal and cataractous soluble proteins[19]. A possible difference between the soluble proteins from the cortex and nucleus of the normal lens was negligible; all protein bands were present in both nucleus and cortex (Figs. 1D and 1E).

A convenient micro method has been developed, and the results are shown in Fig. 3. Four samples were applied: (A) total chick lens crystallins; (B) cow lens cortex crystallins; (C) human lens cortex crystallins (cataractous) and (D) total human lens crystallins (normal). The isoelectric focusing patterns proved to be reproducible also in a small-scale experiment.

The isoelectric focusing method in thin-layer polyacrylamide gel shows very good reproducibility, high sensitivity and extraordinary resolving capacity. The results given by this method provide a new possibility in the study of eye lens proteins, especially in the detection of impurities[15] present in pure lens protein fractions.

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