

## Separation of the Subunits of Denatured Collagens and Gelatins with Starch Gel Electrophoresis

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Gelatinized soluble collagens could be divided into at least six distinct subunits by starch gel electrophoresis. The remnants of these subunits of collagen could be detected also in certain commercial gelatins. At reconstitution some subunits disappeared and a new fraction was demonstrated. The salt-soluble and acid-soluble collagens from rat skin did not differ markedly in their subunit composition, but neutral salt-soluble collagens from lathyratic rats yielded  $\alpha$ -units only.

Gelatins can be fractionated to their subunits by column chromatographic procedures,<sup>1-4</sup> ultracentrifugation<sup>1,5-7</sup> or precipitation<sup>8</sup> and there is evidence that the fractions differ according to the charge and molecular size<sup>3,4</sup>. Therefore a separation of electrophoretic fractions can be expected. Tomlin and Turner<sup>9</sup> studied heat-denatured tendon collagens by free electrophoresis and found that the solutions were not homogeneous. The authors stress the difficulties due to molecular aggregation on cooling.

We first perused a number of conditions: as the support different papers of varying thickness and porosity and also prepared from several ion-exchange celluloses, the substitution of the paper with cellulose acetate sheet "Oxoid", different temperatures from zero to +40°C, variation of pH from 4 to 8, and the suspension of the paper freely or placing it between two insulating sheets. Both commercial gelatins from acid and limed precursors and laboratory samples from various soluble collagens were tried. All the experiments were unsuccessful, because of adsorption of the materials on the support or because of the viscosity of the samples. In retrospect, many of the commercial samples cannot be divided into distinct electrophoretic fractions.

When we learnt that Dr. J. Gross (Massachusetts General Hospital, Boston, Mass., U.S.A.; personal communication) had already in 1961 been successful in separating the  $\alpha$ - and  $\beta$ -components by electrophoresis in polyacrylamide and starch gels, we were encouraged to experiment further with gel supports. In our hands the starch gel in pH 4.5 buffer was the most suitable.

The purpose of this paper is to describe our procedure, to demonstrate some "extra" fractions, the appearance of the residual bands in commercial

gelatins, the effect of lathyrism on the subunit pattern, and, finally, to show the effect of reconstitution on the subunit composition.

### EXPERIMENTAL

*Procedure.* The gel sheet (50 × 110 × 6 mm) was prepared as described by Smithies<sup>10,11</sup> from hydrolyzed starch (Lot 171-1), purchased from Connaught Medical Research Laboratories (Toronto, Canada). The dry material was suspended (11.5 % w/v) into pH 4.5 acetate buffer, ionic strength *ca.* 0.04. The supported gel sheet was connected to the electrode vessels with moist Whatman 3MM paper, using an arrangement described by Kohn<sup>12</sup>. The electrolyte solution was pH 4.5 acetate buffer, ionic strength *ca.* 0.08. A strip of paper was imbibed with the dissolved sample (about 1 mg protein) and placed into a slit (made with a razor blade perpendicularly to the direction of current) in the gel. A voltage gradient of about 15 V/cm was applied. The run lasted 24 h and it was performed in the + 37°C room, as a rule.

The staining procedure was adopted from Vahvaselkä,<sup>13</sup> but Amidoblack was substituted by 0.02 % aqueous solution of nigrosine (from Riedel-de Haën AG, Seelze-Hannover), which stained better all the bands (*cf.* Fig. 1) and which was adsorbed to the fractions more tightly than Amidoblack. Other stainings were also tried, *e.g.* the collagen stain of Heidenhain<sup>14</sup> ("azan"), but they did not seem to offer any advantage.

For the quantitative evaluation the density of the bands was recorded with a Beckman Model B spectrophotometer using an attachment constructed in our laboratory, to make possible the measurement of the transmission through the gel (immersed in glycerol) at 0.1 mm steps (Fig. 2). The absorption maximum of the nigrosine stain was at 6250 Å.

The electrophoretic mobility was calculated from the time interval, the migrated distance and the voltage gradient (measured with Knight Kit Electronic VTVM Y 125 voltmeter). The electro-osmosis was negligible, because in the conditions of the electrophoresis urea diffused evenly to both sides of the application line, which was demonstrated by staining the gel with Ehrlich's *p*-dimethylaminobenzaldehyde reagent.

*Materials.* The laboratory collagens had been prepared by Dr. Tapio Nikkari in our laboratory from the skins of normal and lathyratic rats. The ground rat skin had been extracted with 0.45 M NaCl solution (containing 1/15 M phosphate buffer, pH 7.4). The salt-soluble collagen had been precipitated four times with NaCl (in the final concentration of 16 % w/v), lyophilized and dissolved into 1/12 M acetic acid. This final solution of salt-soluble collagen was cleared in the centrifuge at 100 000 *g* for 60 min at + 5°C. The residue after the NaCl-extraction was re-extracted repeatedly with 0.5 M acetic acid. The combined acetic acid-extracts contained the acid-soluble collagen, which was also centrifuged at 100 000 *g* as above.

These samples were dialyzed against pH 4.5 buffer (ionic strength *ca.* 0.04) and heated for the gelatinization at + 40°C for 15 min.<sup>1</sup> All the samples were stored as frozen solutions but heated at + 40°C for 15 min before the electrophoretic run.

The commercial gelatins have been used in our earlier analyses<sup>3,4</sup> for the fractionation of gelatins. The following samples were now studied:

- I. pI 5.2, from "cartilage and good bones";
- IV. pI 6.9, prepared for bacteriological purposes;
- V. pI 6.8, specially prepared by Kind & Knox Gelatin Co. from pork skin after acidic pretreatment;
- VI. pI 6.8, second extract after V;
- VII. pI 4.95, specially prepared by Kind & Knox Gelatin Co. from limed calf skin;
- VIII. pI 4.9, second extract after VII;
- IX. pI 4.85, third extract after VIII.

*Identification of the bands.* The gelatinized acid-soluble collagen was centrifuged (by Dr. T. Nikkari) in a separation cell of Spinco analytical ultracentrifuge to separate the  $\alpha$ - and  $\beta$ -fractions. The  $\alpha$ -fraction was collected and analyzed electrophoretically as described above. Several fractionations were run also in CM-cellulose columns using the elution with salt strength gradients.<sup>1,2,15</sup> Two main peaks were obtained. The first peak yielded two bands, designated as  $\alpha_1$  and  $\beta_2$  (weak), the former half of the second peak gave a

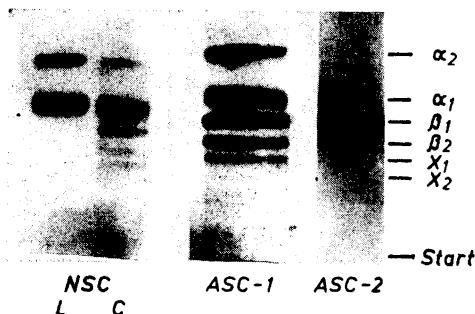


Fig. 1. Typical electrophoretic patterns of acid- and neutral salt-soluble collagens from rat skin, gelatinized for 15 min at  $+40^{\circ}\text{C}$  at pH 4.5. ASC acid-soluble collagen, NCS neutral salt-soluble collagen; ASC-1 stained with nigrosine, ASC-2 the same sheet stained with Amidoblack; C sample from normal rat, L from lathyritic rat. The mobilities of the bands are (averages from three measurements in  $10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1}$ ):  $\alpha_2 = 4.58$ ,  $\alpha_1 = 3.55$ ,  $\beta_1 = 3.10$ ,  $\beta_2 = 2.63$ ,  $X_1 = 2.30$ .

band, believed to be  $\beta_1$ , and the latter half of the second peak the same band ( $\beta_1$ ) but in addition also another band, presumably  $\alpha_2$ .

*Reaggregation.* In this series all the electrophoretic runs were performed at room temperature ( $21-23^{\circ}\text{C}$ ). Much material remained on the starting line. At  $+4^{\circ}\text{C}$  no migration was observed. All the samples were gelatinized at  $+40^{\circ}\text{C}$  for 15 min at pH 4.5. For reaggregation the gelatinized samples were kept at  $+4^{\circ}\text{C}$  for 24 h and thereafter at room temperature for further 24 h. In some experiments the samples were studied immediately after the treatment at  $+4^{\circ}\text{C}$  without the interval at room temperature.

## RESULTS

*Gelatinized soluble collagen.* The typical pattern is shown as Fig. 1, which shows the designation of the bands and the average mobilities. The nature of the peaks designated  $X_1$  and  $X_2$  is not known. In the column fractionation  $X_1$  emerges together with  $\alpha_1$ -fraction. Therefore they may represent breakdown products of  $\alpha$ -chains similar to those obtained by Gallop, Seifter and Meilman,<sup>16</sup> after treatment of collagen with hydroxylamine or hydrazides. This concept is strengthened by the finding that far-degraded commercial gelatins from lime-treated precursors also migrate very slowly. The other relevant possibility would be the  $\gamma$ -unit,<sup>17,18</sup> which contains the three peptide chains of

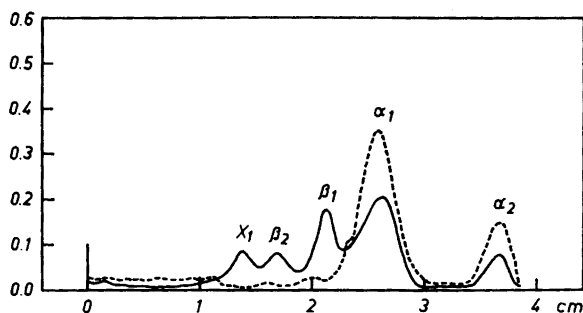


Fig. 2. Densitometric pattern of two strips shown in Fig. 1. The wavelength of  $6250 \text{ \AA}$  has been used. The whole line refers to the sample NSC-C and the dotted line to the sample NSC-L. The abscissa shows the migrated distance in cm, the ordinate gives the extinctions.

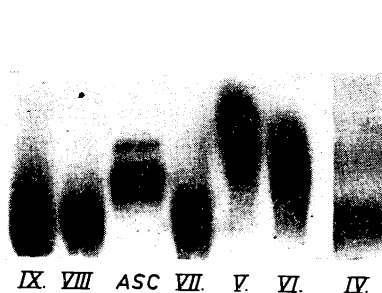


Fig. 3. Comparison of various commercial gelatin samples. Designations of the samples explained in the text. The sample ASC is the reference (ASC-1 shown in Fig. 1). The sample IV was run in a separate sheet.

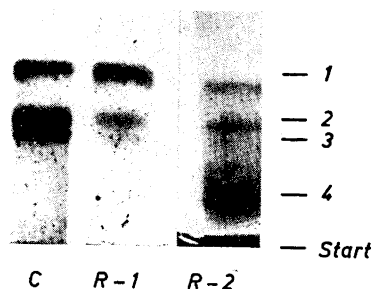


Fig. 4. Effect of reaggregation. C control sample (ASC-1 in Fig. 1), studied by electrophoresis at room temperature immediately after gelatinization for 15 min at + 40°C at pH 4.5, R-1 the same sample reaggregated at + 4°C 24 h and then at room temperature 24 h, R-2 reaggregated 24 h at + 4°C only. Note the fraction 4 in R-2.

collagen still bonded together. In fact, it is not proved, that  $X_1$  and  $X_2$  are gelatins at all. Fig. 2 shows the densitometric pattern of the sample shown in Fig. 1.

*Commercial gelatins.* A comparison of several samples is given in the Fig. 3, which shows that in some gelatins clearly defined subunits are still left. One or two bands can be seen in most freshly stained electrophoretic patterns, but they are difficult to reproduce in photographs. However, the bulks contain a large dispersion of related fractions. It is not known how much the varying molecular size and charge contribute to this dispersion.

The mobility of gelatins from acid precursors is larger than of samples from limed precursors, which indicates that they are more basic, presumably because of the more numerous amide groups which are left.

*Effect of lathyrism.* The comparison of salt-soluble collagens of normal and lathyrinic rats is shown in Fig. 1. The finding confirms the previously shown abundance of  $\alpha$ -fractions in the lathyrinic samples<sup>19-20</sup>. Only faint  $\beta$ -bands are present in the patterns from lathyrinic samples.

*Effect of reaggregation.* Fig. 4 shows the bands which were obtained from samples which had been allowed to reaggregate at + 4°C. Also the control, run at the room temperature, differed from that obtained at + 37°C. After the reaggregation the pattern changed further, but the significance of the new bands was not investigated.

## DISCUSSION

The separations which can be obtained with the electrophoretic method are superior to column chromatography, ultracentrifugation or precipitation in regard to sharpness of resolution and amount of work required. A drawback is the small capacity. Work for a preparative modification is in progress.

Preliminary experiments have shown that instead of heating, the denaturation can be performed with urea at room temperature, and thus the effect of possible reaggregation can be eliminated.

It seems that the technical properties of the commercial gelatins have no bearing with the electrophoretic pattern, which is more influenced by the pretreatment. In any case, it would be interesting to know the properties of a gelatin, containing for example only pure undegraded  $\alpha_1$ -components etc. By choosing a suitable pH for the electrophoretic fractionation the bulk of the related fractions could be divided according to the isoelectric points of the fractions. The separations of commercial gelatins obtained with Amberlite or carboxymethyl cellulose columns<sup>3,4</sup> also obviously depend on the selection of the pH of the buffers as suggested earlier.

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