COMBINATION OF TEMPERATURE GRADIENT WITH GEL ELECTROPHORESIS AND ITS APPLICATIONS TO ANALYSIS OF COLLAGEN–GELATIN TRANSITION

T. HOLLMÉN AND E. KULONEN
Department of Medical Chemistry, University of Turku, Turku (Finland)
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INTRODUCTION

When the effect of temperature on the electrophoretic migration of denatured collagen was studied[1], the denaturation of collagen and the reconstitution of gelatin could be evaluated from the starch-gel electrophoretic patterns. In appropriate conditions native collagen and reconstituted gelatin do not migrate at all whereas unfolded gelatins are separated into their subunits. The conditions of the reconstitution seemed to be different for the various subunits.

To develop a reproducible, fast and simple method for studying the collagen–gelatin transition of different collagens and collagen subunits, a device was constructed for the combination of a temperature gradient with starch-gel electrophoresis. The linear temperature gradient is created by thermal conduction in an aluminium block between the opposite edges, which are maintained at two different, constant temperatures.

APPARATUS

Temperature gradient device

A constant temperature difference is maintained between the sides CD and C'D' of a prism-shaped metal block (Fig. 1), which is thermally insulated from the surroundings. The thin gel sheet is pressed against the bottom surface (AA' and BB') of the block and acquires its temperature gradient.

The block was made of cast aluminium shaped to the following dimensions: 30 mm × 145 mm × 150 mm. Two chambers (22 mm × 131 mm wide and 10 mm deep) were machined one to each side of the aluminium block. The chambers were closed with brass plates, using machine screws and rubber and polythene gaskets. Nozzles (C, C', D, D') were provided for connection to pumps which circulated water from two thermostats.

For thermal insulation, all sides of the block except the bottom surface in contact with the gel were coated with a 20 mm thick layer of plastic foam, "Styrox" (E).

Electrophoretic system

The gel trough[2] had an exchangeable bottom plate allowing the thickness of

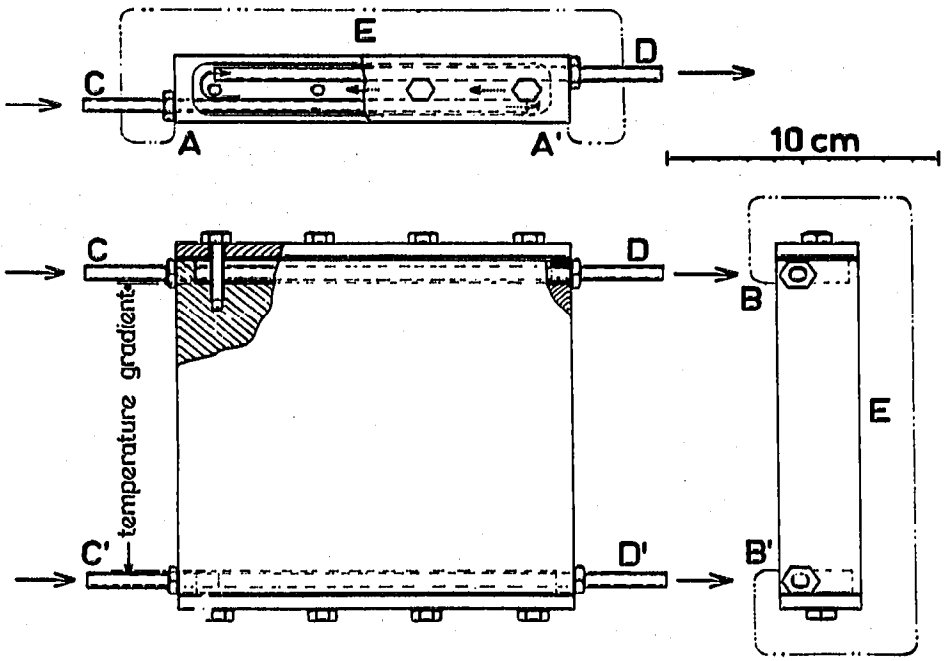

Fig. 1. Temperature gradient device. AA′ = surface against the gel sheet, in the direction of the migration; BB′ = surface against the gel sheet, perpendicular to the migration; C and C′ = nozzles for inflow of the water from thermostated vessels; D and D′ = nozzles for the corresponding outflow; E = insulating layer of plastic foam.

the gel to be varied. In the experiments presented here sheets 6 mm thick were used. The other dimensions of the gel sheet were 110 mm × 110 mm. The gel sheet was connected to the electrode vessels by extensions of the gel[2]. The gel trough was also insulated by means of a layer of "Styrox" placed underneath.

The electrode vessels (80 mm × 120 mm × 250 mm) were made of "Perspex". They were each filled with 2.2 l of buffer. A platinum wire was used as the anode and a stainless steel wire as the cathode.

A 0–250 V/0–100 mA continuously adjustable condenser-stabilized selenium rectifier unit was used as the D.C. power supply. The voltage and the current across the electrodes were recorded.

For the electric insulation between the gel and the aluminium block surface and to prevent the gel from drying, the gel was covered on top with two membranes of polythene, each 0.035 mm thick. To ensure a good conduction of heat between the gel and the block, meticulous care was taken to get the gel surface absolutely smooth. Some transformer oil was sometimes applied between the two polythene sheets if a good contact was not achieved otherwise.

EXPERIMENTAL

Collagen samples

Rat tail tendons were dissected in the cold room, washed with cold distilled water and extracted for 36 h with 100 volumes of 0.5 *M* acetic acid at + 4°, with stirring. The extract was centrifuged with a MSE refrigerated centrifuge at 17000 *g* for 120 min at + 4°, the supernatant was lyophilised and stored at — 20°. The dry

lyophilisate was dissolved in the electrophoresis buffer, approximately 8 mg/ml, and the solution was clarified by centrifugation as above.

Buffer and gel

The electrophoresis of collagen was performed under the conditions of optimal resolution[2], using acetate buffer pH 4.7, ionic strength 0.017, in a gel containing 22 g of hydrolysed starch (Connaught Research Laboratories Ltd., Toronto) in 150 ml of buffer.

Electrophoretic runs

The denaturation and reconstitution histories of the samples varied according to the aim of each experiment, and they are given in the legends of the Figs. 2–5.

The sample was absorbed into a 5 mm × 109 mm strip of Whatman No. 3 MM chromatographic paper, which was then inserted into a slot made across the whole width of the gel with razor blades, approximately 3 cm from the anodic end of the gel sheet. Care was taken not to damage the smooth surface of the gel during the insertion of the sample. The sample was applied in a cold room into a cold gel when the denaturation of native collagen was studied, and at + 37° when the reconstitution of denatured collagen was to be followed.

The gel was then covered with the two polythene membranes, and the gradient block, which had already been stabilized to the required temperature gradient, was pressed against the gel. In some experiments, when the time factor was critical, the gel was also stabilized previously to the gradient in the block, which was pressed against the gel again immediately after the sample application. Guide pins were used so that the position of the block on the gel trough was exactly reproducible. The equilibration times under the temperature gradient before the connection of the voltage varied according to the experiment as explained in the legends of the figures.

The voltage was selected so that the production of the ohmic heat in the gel never exceeded 1 % of the flow of thermal energy in the aluminium block, which was calculated to be 6.44 W for each degree in the temperature difference between the flow chambers. The running time was selected to give a migration distance of 2–5 cm for the fastest or α_2-component. The other conditions of electrophoresis are indicated in the legends of the figures.

Water circulation in the flow chambers was achieved by pumps from constant temperature water baths (± 0.1°). An ice-bath was used to give the temperature 0°.

RESULTS AND DISCUSSION

Examples of the potentialities of this method in collagen chemistry are presented in Figs. 2–5 and the exact experimental conditions are given in the legends.

In the studies on the reconstitution of denatured collagen (Figs. 2–3) it appeared that each subunit reconstitutes at a characteristic rate dependent on temperature even when in a mixture of different subunits. This was shown for pure subunits by PIEZ AND CARRILLO[3], who employed viscometric, polarimetric and light scattering methods.

The gelatin molecules which have been driven electrophoretically into the gel cannot fold there any longer, presumably because of steric hindrance and frictional stress.

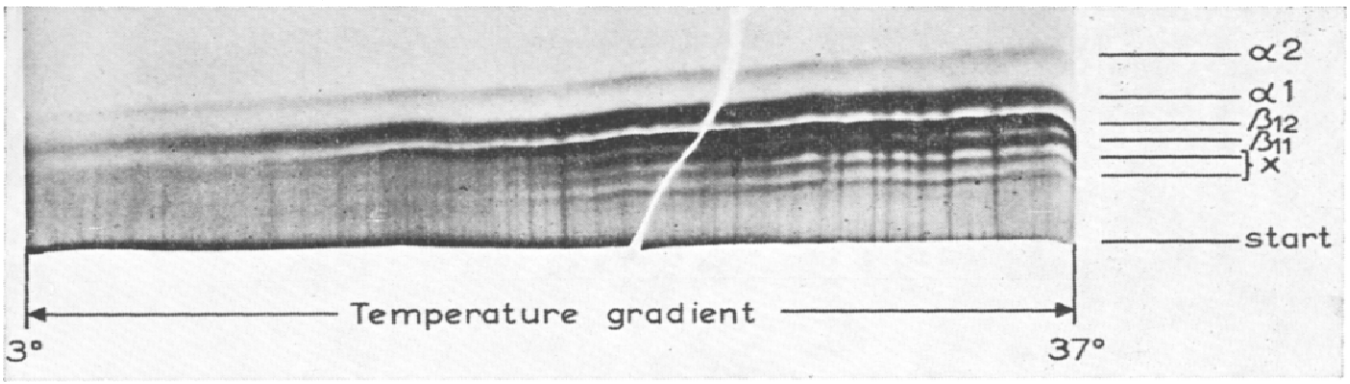

Fig. 2. Reconstitution of denatured collagen I. The collagen sample was denatured for 10 min at + 40° and absorbed on a paper strip, which was inserted in the gel. The reaggregation was allowed to take place for 30 min at a gradient 3–37° (from left to right) before a voltage of 100 V (32 mA) was applied for 6 h while the same temperature gradient was maintained. The dark part on the left hand side of the starting line indicates the reconstituted material which has not migrated at the low temperatures.

This was shown by an experiment where the temperature gradient in the gel was applied in the direction of the current and several short paper strips with the absorbed sample were inserted at points of different temperatures in the gradient. The band pattern of each sample was consistent with the temperature at the application site and with the time interval before connecting the voltage. The migration rates of the unfolded molecules (gelatins) did not change when they reached lower temperatures during the run, nor was there any tailing or blurring of the bands.

The reconstitution of subunits in denatured collagen as tested by this method seems to be an all-or-nothing process, because the fading ends of the bands are not curved at all and there is no tailing or blurring of the bands. The molecules presumably either aggregate together at folding, or the folding of even a part of the peptide chain

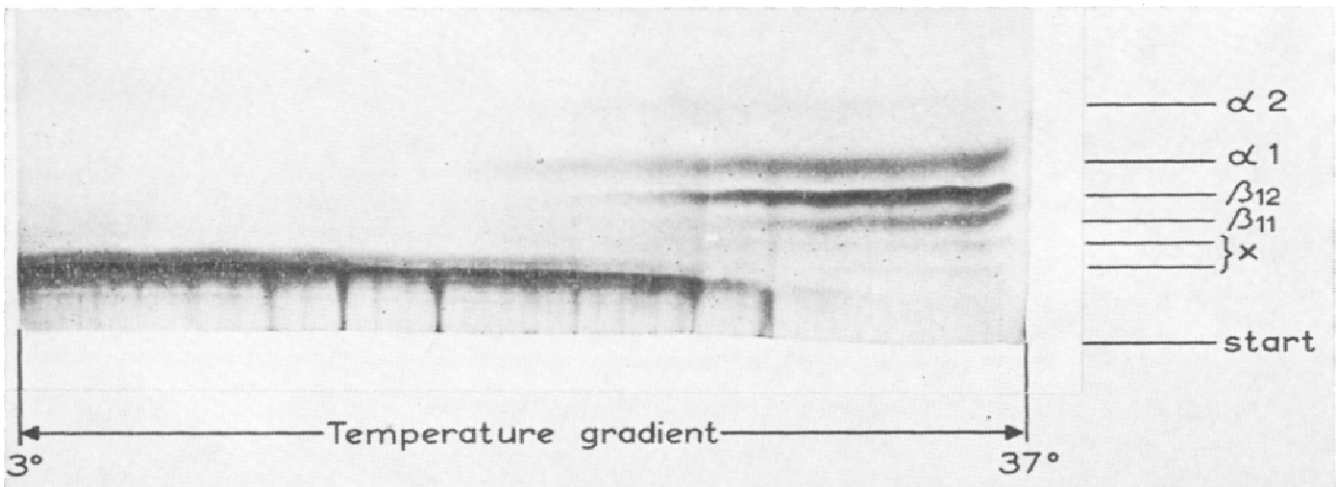

Fig. 3. Reconstitution of denatured collagen II. The collagen sample was denatured for 30 min at + 40° and allowed to reconstitute for 60 min in the gradient 3–37° (from left to right). The first electrophoretic run was carried out at 80 V (25 mA) for 6 h in the gradient. The second run (8 h at 40 V and 15 mA) followed after a further denaturation by raising the temperature in the whole gel to + 40°. The voltage was connected after 30 min. The second run was made for the analysis of the material which had been retained at the starting line during the first run (*cf.* Fig. 2).

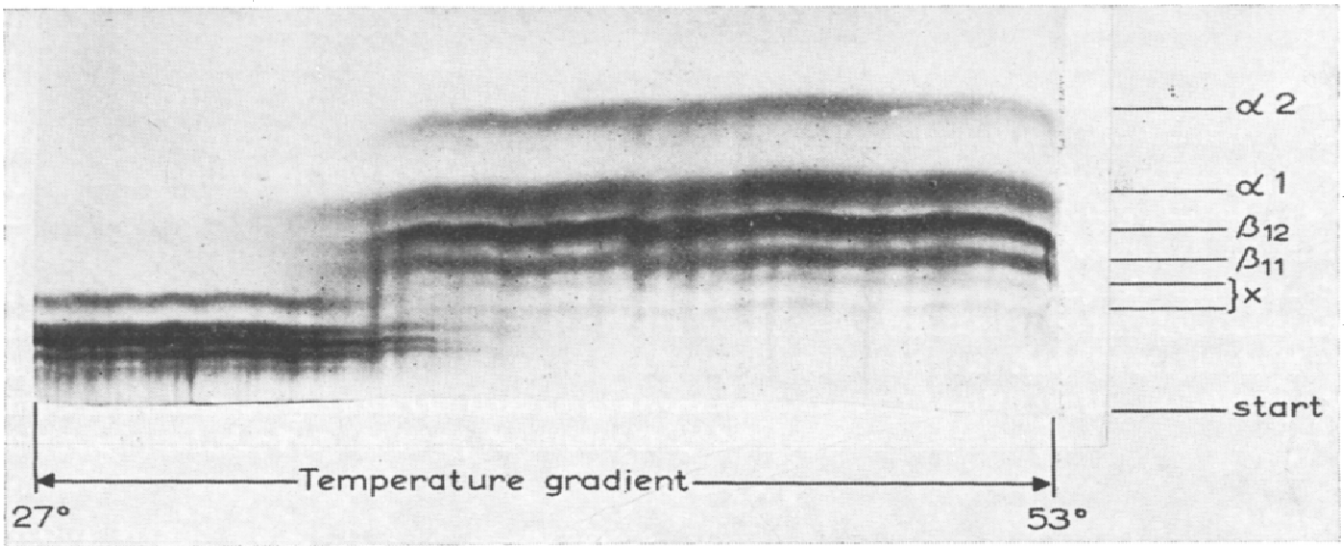

Fig. 4. Denaturation of native collagen. The sample of soluble collagen was inserted in the gel which had previously been equilibrated to the temperature gradient 27–53° (from left to right) and the gradient maintained for 30 min before the first electrophoretic run at 60 V (23 mA) for 8 h. The temperature of the whole gel was then raised to + 45° for 90 min and a second run was carried out at 60 V (23 mA) for 8 h at + 45°, analogously to the experiment shown in Fig. 3. The sharp denaturation of tropocollagen molecules at + 35.5° is seen (*cf.* the variable reconstitution temperatures of the subunits, Figs. 2 and 3).

increases the dimensions of the molecule so that it cannot pass the pores of the starch-gel molecular sieve any more.

In the studies on the denaturation of native collagen this method gives the final equilibrium state after a long denaturation time[4]. If the equilibration time before the beginning of the electrophoretic run is too short, some tailing is observed. The present criterion of denaturation, *i.e.*, the appearance of subunits, differs from those observed in the studies employing the classical methods, and the transition tempera-

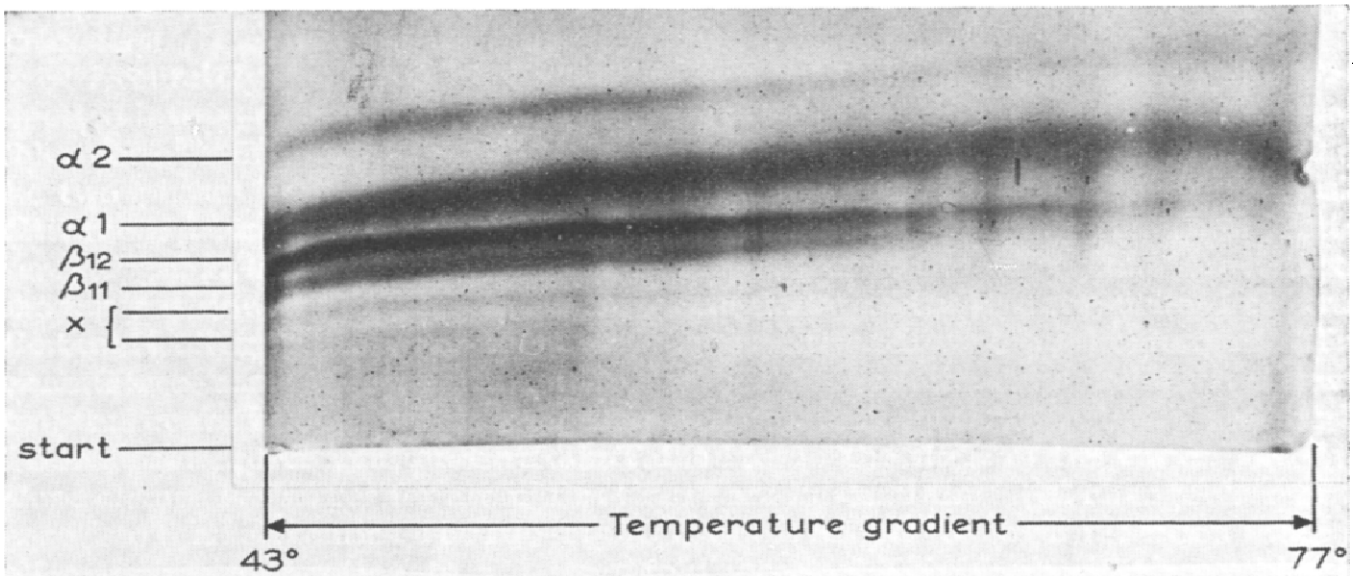

Fig. 5. Thermal degradation of native collagen. The sample of soluble collagen was denatured for 30 min in the gradient 43–77° (from left to right) before the run at 40 V (19 mA) for 19 h in the same gradient. The progressive loss of the distinct band pattern, especially of the x- and β-fractions, is observed.

tures obtained by different methods should be compared with some caution. Preliminary observations suggest that even small differences can be shown by the present method between the denaturation temperatures of tropocollagen molecules with varying cross-linking.

This method has several advantages in the study of the temperature-linked changes in collagen. The procedure is easy, simple and fast. In contrast to the classical methods, every component in a mixture is analysed separately without any preceding fractionation process. The band pattern is conspicuous and its continuity through the temperature range renders it easy to assign the bands in spite of slight differences in the mobility. Because all the different temperatures are comprised on the same gel sheet, the occurrence of misleading artefacts is decreased.

The main drawbacks of the method are that it is less accurate than the classical methods and that the times and concentrations cannot be so well controlled. The presence of starch in the medium may affect the properties of the materials being studied. The choice of the ionic environment is also restricted[2].

The temperature gradient block can be also used for other purposes. The combination of a temperature gradient with the different "thin layer"-separation methods might be rewarding; for example, when the optimal temperature for a given separation is sought or when temperature-dependent processes are studied by "thin layer"-methods.

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SUMMARY

An apparatus is described for creating a temperature gradient for the combination with starch-gel electrophoresis or with "thin-layer" type separation methods.

The device is applied to studies on the reconstitution and thermal denaturation of collagen.

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