PRO EXPERIMENTIS

A rapid electrophoretic method for the separation of hydroxyproline from proline[1]

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Summary. A rapid, sensitive procedure is described which uses custom made apparatus to separate radioactive imino acids. This technique provides a simple measurement of the distribution of newly synthesized collagen in different fractions.

Radioactive hydroxyproline (hyp) and proline (pro) were separated from hydrolysates of fractions from chick embryos by high voltage paper electrophoresis in order to compare the relative amounts of newly synthesized collagen.

Collagen can be considered unique in that about half the pro residues are hydroxylated post-translationally. Thus the amount of new collagen synthesis following a period of labeling with radioactive pro is related to the amount of radioactive hyp formed. Since the hydroxyl group is added mainly in the 4-position of pro, the use of pro tritiated in the 5-position ensures that the ^{3}H-label is retained after hydroxylation.

Pro and hyp have previously been separated on ion exchange columns but the procedure takes longer than 4 h and only 1 sample per column can be run at any one time[2]. An electrophoretic method is described here which is faster and more sensitive than column techniques.

Chick embryos were labeled with [^{3}H] pro and the relative amounts of radioactive hyp and pro in fractions produced by differential centrifugation were determined. Fractions were precipitated with trichloroacetic acid (TCA), hydrolyzed and the amino and imino acids separated by high voltage paper electrophoresis (HVE). Strips of paper were dried, cut into pieces, and counted directly by a liquid scintillation system.

Materials and methods. The reagents used were of analytical grade (Analar, British Drug Houses, Poole, England); L-proline and L-hydroxyproline imino acid standards were obtained from Fluka (Switzerland). Tritiated pro labeled in the 5-position (L-proline-5-^{3}H, 10 Ci/mole) was obtained from Radiochemical Centre, Amersham (England). pH 1.85 buffer was made by diluting 78.0 g of formic acid and 25.0 g of acetic acid to 1 litre with water. The homogenizing buffer RSB contained 0.01 M Tris, 0.01 M NaCl and 0.0015 M $MgCl_2$, pH 7.4. Isatin reagent was made by dissolving 1.0 g of isatin, 1.5 g of zinc acetate and 1.0 ml of pyridine in 100 ml of isopropanol and warming at 80 °C for 30 min.

The HVE apparatus was constructed in the workshops of the Wool Industries Research Association, Leeds, and is based on Michl's design as modified by Gross[3,4] and described by Atfield and Morris[5] (figure 1).

The paper strip (Whatman 3MM filter paper 115 cm × 15 cm) is held horizontally between 2 water-cooled, anodised, aluminium-copper alloy plates and insulated from them by 2 strips of thin polythene sheet. The cooled plates and insulating polythene sheets are held in close contact with the paper by uniform pressure applied by an inflated rubber bladder above the upper plate. The bladder is inflated by a compressor. Each electrode comprises a Perspex (or Plexiglass) box containing pH 1.85 buffer into which a platinum terminal dips. The circuit is completed by joining the paper to the buffer with paper wicks, the same width as the paper. The whole assembly is housed in a Perspex cabinet that incorporates a safety switch so that the electrodes can only be made live when the window is closed. The power-pack provides potential differences up to 20 kV thus giving potential gradients of 190 v/cm for paper strips 105 cm long.

Preparation of samples. 10-day-old chick embryos were labeled in ovo with 100 μCi of ^{3}H-pro in 0.1 ml of water. The shell covering the air space was removed and the embryos injected in the thorax using a 30 gauge needle. They were incubated a further 25 min before decapitating and homogenizing in 5 ml of RSB each. 5 fractions were prepared and rinsed with 7% TCA as described in table 1. The precipitates were hydrolysed with 6 N hydrochloric acid at 110 °C for 20 h in sealed glass tubes. The hydro-

Table 1. The centrifugation conditions used to prepare a series of 5 pellets SP1–SP5

Fraction number	Fraction removed after	g
SP1	2,000 rpm, 10 min, SW39	325 × g
SP2	5,000 rpm, 15 min, SW39	2040 × g
SP3	15,000 rpm, 15 min, SW39	18,400 × g
SP4	35,000 rpm, 30 min, SW39	100,000 × g
SP5	Supernatant precipitated with trichloroacetic acid	–

1 10-day-old chick embryo was homogenized in 5 ml of RSB with 10 strokes of a loose Dounce pestle. The homogenate was centrifuged as indicated and the pellets retained at each stage. The pellets were rinsed twice with 5 ml of 0.1 M unlabeled pro in 7% TCA and recentrifuged at 90,000 × g for 15 min.

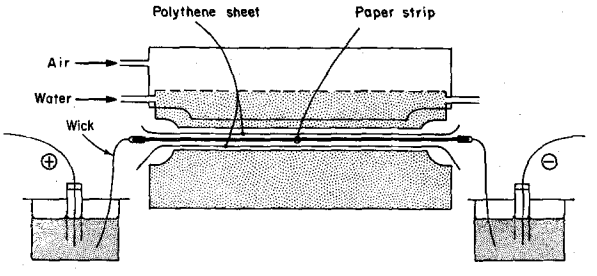

Fig. 1. High voltage paper electrophoresis apparatus.

lysates were centrifuged at 170,000 × g for 20 min to remove charred debris and dried over solid NaOH under vacuum. The amino and imino acids were dissolved in pH 1.85 buffer and applied directly to HVE paper.

The paper strip was dipped in pH 1.85 buffer and excess liquid expressed by passing through a twin rubber roller mangle adjusted to such a pressure that the paper retained its own weight of electrolyte. Hydrolysate was then applied as a small spot or fine line. Loads of up to 20 µl could be applied as fine lines 1 or 2 cm long. The prepared strip was placed in position between the 2 polythene insulators on the lower metal plate and connected to the 2 electrode wicks. The upper plate was lowered and secured and the pressure bladder inflated to ensure good thermal contact. The cooling water was switched on, the Perspex window closed and the voltage raised to the required value. Between 14 kV and 16 kV was used, normally for 55 min.

After the electrophoretic separation, the paper was removed and dried in a heated cabinet. The positions of the amino and imino acids were determined by cutting off duplicate sample strips 2 cm wide and spraying with isatin reagent. Pro and hyp show up as green spots and the amino acids show up as fainter brown spots against the background of bright orange. The remaining unstained samples were counted directly with 0.4% diphenyl oxazole in toluene. Stained samples were not counted because of the increased chemical and colour quenching produced.

Results. Figure 2 shows the electrophoretic separation of hydrolysates of the fractions SP1–SP5. The positions of the imino acids were determined by running duplicate samples containing 0.05 µmole of unlabeled hyp and pro and staining with isatin reagent.

The small and large peaks of radioactivity coincided exactly with the positions of the hyp and pro standards, respectively. The electrophoretic mobilities observed were 0.54 for hyp and 0.69 for pro relative to alanine as 1.00. The total radioactivity associated with each imino acid is shown in table 2.

Although the slow speed pellet, SP1 contains 49–50% of the pro radioactivity, 60% of the radioactive hyp, and therefore newly synthesized collagen polypeptides, was present. The remaining hyp radioactivity was distributed between fractions SP2–SP5 with each fraction containing less than 14% of the total radioactive hyp.

Discussion. The procedure described is a rapid, sensitive method of separating radioactive hyp from pro. The technique provided a simple measurement of the distribution of newly synthesized collagen and procollagen chains in fractions prepared from 10-day chick embryos.

Up to 5 samples of 5–25 µg of hydrolysate can be run at any one time which is approximately 5–10 times more sensitive than ion exchange column techniques. Furthermore the separation can be completed and paper strips dried and counted in less than 100 min offering considerable time savings. By using different buffer pHs 18 amino acids can be separated and determined quantitatively if required by reaction with cadmium-ninhydrin[5]. For the determination of hyp and pro, a cadmium-isatin reagent is more sensitive. The chromophore is extracted with methanol and the extinction measured at 352 nm for hyp and pro (610 nm if cadmium-isatin reagent is used).

Table 2. Total hyp and pro radioactivity from the separations of hydrolyzed SP1–SP5 shown in figure 2.

Vol. of hydrolysate (µl)		Vol. loaded (µl)	Sum of counts associated with hyp and pro peaks		Total for each fraction		hyp/pro ratios
			hyp	pro	hyp (x 10^{-3})	pro (x 10^{-3})	
SP1	250	20	2,990	39,590	37.4	495	0.076
SP2	250	20	470	4,586	5.87	57.3	0.102
SP3	250	20	464	4,610	5.80	57.6	0.101
SP4	250	20	231	5,691	2.89	71.0	0.041
SP5	250	20	668	25,618	8.35	320	0.026

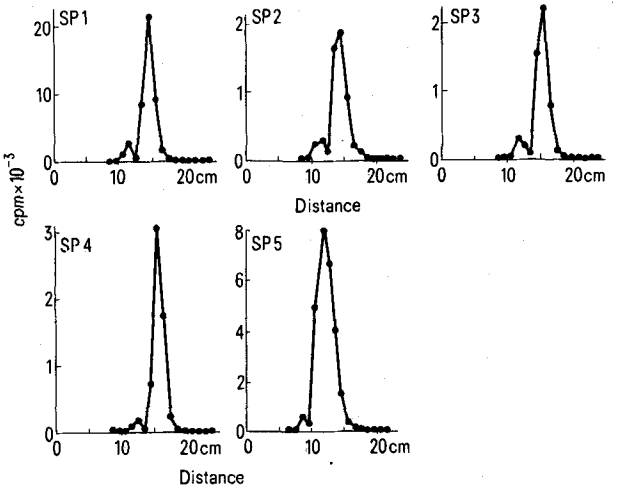

Fig. 2. Distance versus radioactivity plots for HVE paper from the separation of hydrolyzed fractions SP1–SP5. Each pellet was hydrolyzed in 2 ml of 6 N HCl, the hydrolysates were dried over solid NaOH and then redissolved in 250 µl of pH 1.85 buffer. Loads of 20 µl were applied and run at pH 1.85, 14 kV, 55 mA for 55 min. ●—●, radioactivity associated with pieces of HVE paper 2 cm × 1 cm.

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