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Note

An accelerated single column procedure for the programmed analysis of naturally-occurring amino acids of collagen and basement membrane*

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Several procedures have been devised for the accelerated analysis of complex amino acid mixtures of collagen hydrolysates. These procedures basically differ in the use of a single-column system with continuous buffer gradient elution[1,2] or a two-column system with stepwise buffer elution[3–5]. A single-column system for the complete analysis of acidic, neutral, and basic amino acids possesses the advantages of procedural simplification, reduction in the amount of sample required, and a decreased chance of error. However, the present single-column systems for analysis of collagen hydrolysates[1,2] do not permit the use of commercially available multi sample programmers which is based on the stepwise buffer elution principle.

This paper describes an accelerated single-column procedure for the analysis of collagen and basement membrane hydrolysates utilizing a multi sample programmer which automates stepwise buffer changes, temperature changes, regeneration, equilibration, sample injection, and final shutdown after a programmed series of up to nineteen consecutive samples. This procedure completely resolves all the components present in collagen hydrolysates as well as those in the slightly more complex basement membrane hydrolysate. The latter hydrolysate contains glucosamine and galactosamine[6] and S-carboxymethylcysteine in reduced and alkylated membrane[7] in addition to the typical collagen components.

The amino acid analyses were performed on a Beckman (Fullerton, Calif., U.S.A.) 120C instrument equipped with a Bio-Cal (Rockville Center, N.Y., U.S.A.) AS-20 multi sample programmer[8]. The standard long column was packed to a column height of 54 cm with Beckman resin Type AA-15. A stepwise 4-buffer elution program, which is related to the system described by Dus *et al.*[9] was devised to separate all the components. The preparation and composition buffers are given in Table I and the elution program in Table II. Flow-rates were maintained at 70 ml/h for the buffers and 35 ml/h for the ninhydrin. The ninhydrin reagent was prepared as described in the Beckman manual except titanous chloride was substituted for stannous chloride. Calibrations were performed with a standard mixture of eighteen amino acids from Pierce Chemical Co. (Rockford, Ill., U.S.A.), to which was added D,L-cysteic acid and S-carboxymethyl-L-cysteine (Mann Research Labs, New York, N.Y., U.S.A.), D,L-methionine sulfoxide and D,L-*allo*-

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TABLE I

BUFFER COMPOSITION

Sodium citrate Beckman concentrates were diluted 1:10. Pentachlorophenol (0.4 ml of stock solution[10] per 4 l of solution) was added as an anti-mold agent. Sodium ion concentrations were adjusted by the addition of crystalline NaCl. Methyl cellusolve and *n*-propanol were added as indicated and the final pH's adjusted with either concentrated HCl or 50% NaOH as required.

Buffer	*Beckman concentrates*		*Final composition*		
	$[Na^+]$	*pH (25 °)*	*NaCl (g/4 l)*	*pH (25 °)*	*Vol.% organic solvent*
A	2.0 *N*	3.25±0.01		2.77	2% methyl cellusolve
B	2.0 *N*	3.25±0.01	7.0	3.29	
C	2.0 *N*	3.25±0.01	30.5	3.61	
D	3.5 *N*	5.26±0.02	292.2	4.70	16% *n*-propanol

TABLE II

ELUTION PROGRAM

Buffer	*Pumping time (min)*	*Run time (min)*	*Column temperature* (°C)*
A	40	0–40	55
B	60	40–100	67
C	55	100–155	67
D	60	155–215	67
NaOH	11 (regeneration)	215–226	67
A	60 (equilibration)	226–286	55

* The column temperature reaches 67 ° 35 min after buffer B has been started, and returns to the initial 55 ° after the regeneration step has been concluded.

hydroxylysine (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.), hydroxy-L-proline (Calbiochem, Los Angeles, Calif., U.S.A.) and D,L-norleucine, D-glucosamine and D-galactosamine (Sigma, St. Louis, Mo., U.S.A.). Ninhydrin and titanous chloride were purchased from Piercé Chemical Co. Bovine glomerular basement membrane, S-carboxymethyl derivative was prepared by the method of Hudson and Spiro[7] and hydrolyzed with 6 *N* HCl in evacuated tubes for 24 h at 110°.

The separation of standard mixture of the amino acids usually found in collagen or basement membrane is shown in Fig. 1. Tryptophan, not shown, elutes at 208 min. A typical chromatogram of the hydrolysate of glomerular basement membrane, reduced and S-carboxymethylated, is shown in Fig. 2. Excellent resolution was achieved for all components in the standard mixture and membrane hydrolysate. The stepwise 4-buffer elution program provides the flexibility to position components in a given region without sacrificing the separation of those in another region. Hence, it was possible to separate widely hydroxyproline from aspartic acid in order to position S-carboxymethylcysteine between the two without sacrificing

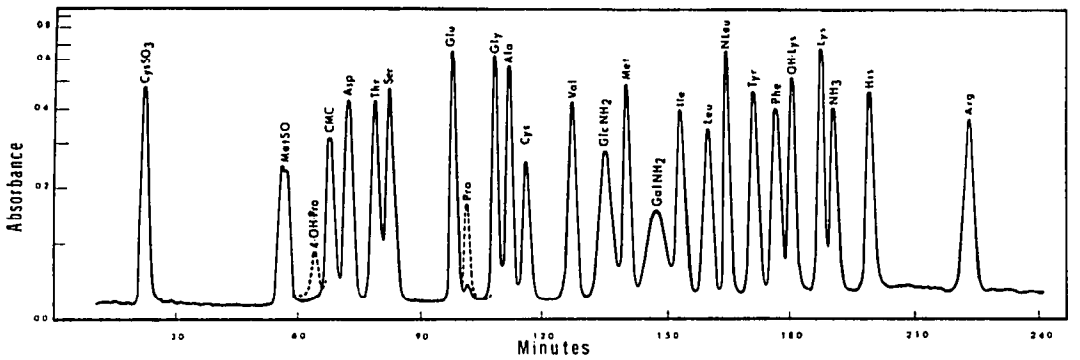

Fig. 1. Analysis of standard mixture of 0.15 μmole of each component. ——, Absorbance at 570 nm, 6.6 mm pathlength; – – – –, absorbance at 440 nm, 6.6 mm pathlength.

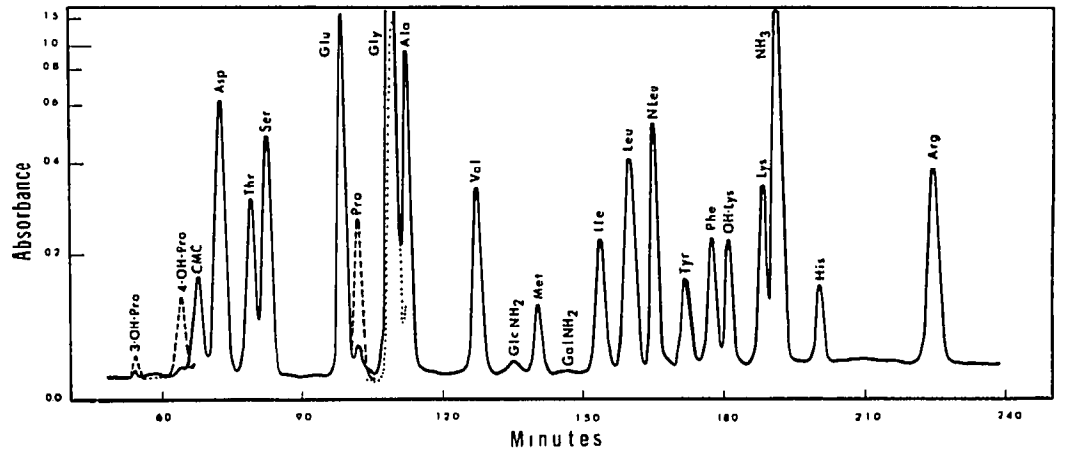

Fig. 2. Analysis of 0.5 mg of bovine glomerular basement membrane S-carboxymethyl derivative ——, Absorbance at 570 nm, 6.6 mm pathlength; ····, absorbance at 570 nm, 2.2 mm pathlength – – – –, absorbance at 440 nm, 6.6 mm pathlength.

the separation of glucosamine and galactosamine from valine and methionine. The hexosamines are not quantitated from these membrane hydrolysates because of large amounts of destruction during hydrolysis, however, it is necessary to resolve the residual amounts of these monosaccharides in order to determine an accurate amino acid composition. This procedure may be used to quantitate glucosamine and galactosamine released from the protein under milder conditions of hydrolysis than those used for normal amino acid analysis. Moreover, the use of the long column permits high resolution of hexosamines from interfering peptides produced from the mild hydrolysis.

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