Journal of Chromatography, 115 (1975) 612–615

CHROM. 8633

Note

Ion-exchange chromatography of the more basic amino acids of protein hydrolysates using stepwise changes of buffers containing borate ions

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(First received May 22nd, 1975; revised manuscript received August 14th, 1975)

Improved separation of basic amino acids and ammonia resulted from replacing sodium phosphate with sodium borate in the gradient elution system of ion-exchange chromatography described by Murren *et al.*[1]. This observation prompted studies into the use of borate-containing buffers in an apparatus which uses stepwise changes of column eluting buffers[2] with equally rewarding results.

EXPERIMENTAL

The modular apparatus used was constructed in the author's laboratory and is described in detail elsewhere[2]. All experiments were performed on a 300 × 6 mm I.D. column of cation-exchange resin (12 ± 1 μm beads, 8% DVB) at a temperature of 55° and an elution flow-rate of 0.6 ml/min (ref. 2). Buffer compositions are given in Table I. Nitrogen-segmented, ninhydrin/hydrazine reagent was used for detection[3].

RESULTS AND DISCUSSION

Whilst maintaining the first two sodium citrate buffers commonly used for the elution of acidic and neutral amino acids, various borate-containing buffers were used to resolve the more basic substances. Solutions of sodium borate used for the third and fourth buffers produced irregularly shaped peaks which regained a gaussian form if both citrate and borate ions were present in the buffer. If the pH of the third buffer was greater than 7.20, tyrosine and phenylalanine were not separated; and if the buffer change was made after the emergence of phenylalanine, a pH greater than 9.00 was required to elute (in an acceptable period of time) the amino acids which follow histidine. A third buffer of pH 10.50, introduced after the elution of phenylalanine, resolved histidine, lysine, ammonia and arginine in 12 min (Fig. 1) but tryptophan emerged with lysine. Acid-hydrolysed samples could be analysed at a rate of one every 110 min with this system.

A system using four buffers is shown in Fig. 2 and described in Table I, and was preferred since tyrosine and phenylalanine emerged as sharp peaks, tryptophan was separated from lysine, and there was less likelihood of gross amounts of ammonia overlapping lysine or arginine[1]. Either norleucine or anisylalanine[3] could be used as

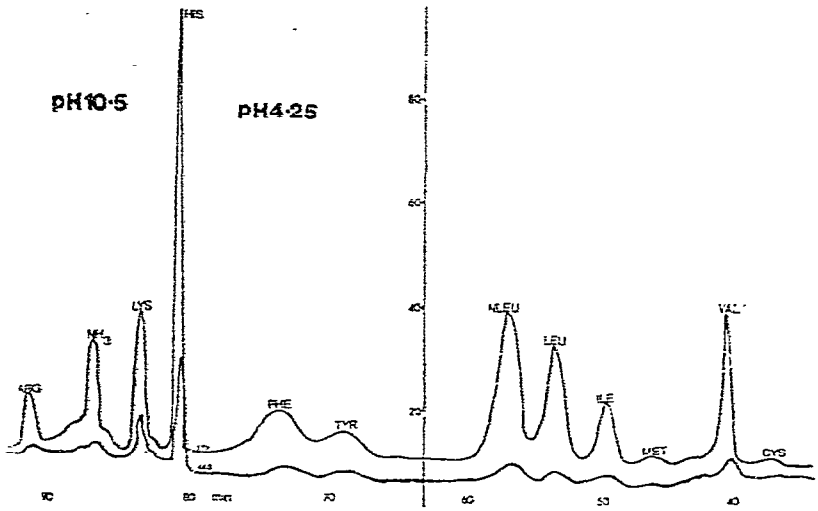
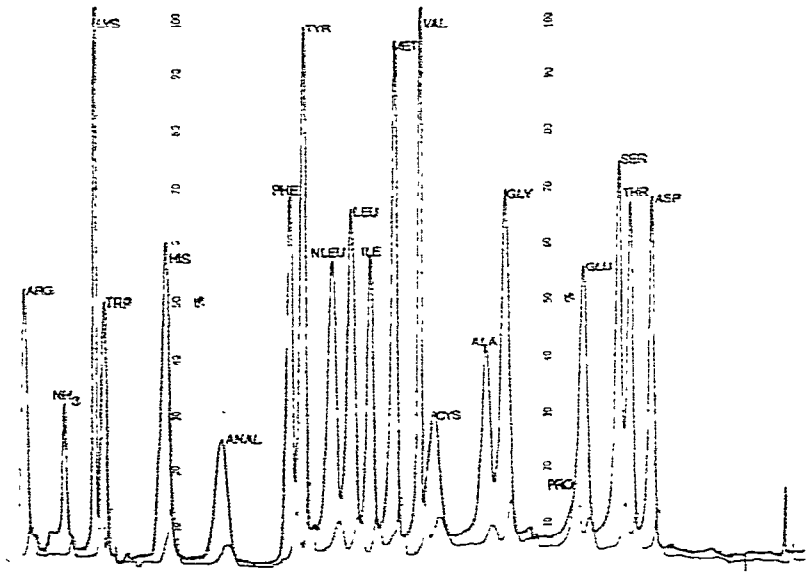


Fig. 1. Latter portion of a chromatogram of acid-hydrolysed bean meal (*Phaseolus vulgaris*).

internal standards. A complete analytical cycle took 132 min (Table I) and this could be shortened to 128 min if anisylalanine was used as an internal standard, when the second buffer change (Step 4, Table I) could be advanced by 4 min.

The observations of Thomas[4] and Murren *et al.*[1] that there was little shift in recorder baseline if buffers of increasing pH, rather than greater molarity, were used to elute basic amino acids, were confirmed (Figs. 1 and 2) provided the ninhydrin had sufficient buffering capacity. Slight increases in the baseline of the 440-nm



ig. 2. Chromatogram of a standard mixture of amino acids separated with the elution programme iven in Table I. 20–50 nmoles of each substance were present in the mixture. ANAL = Anisylalanine internal standard).

TABLE I

ELUTION PROGRAMME FOR THE ANALYSIS OF PROTEIN HYDROLYSATES USING FOUR BUFFERS

Position	*Time (min)*	*Buffer**	*Functions*
1	15	8 g sodium hydroxide, 21 g citric acid monohydrate and 60 ml 2-methoxyethanol; pH 3.25	Column regeneration
2	43	8 g sodium hydroxide, 21 g citric acid monohydrate and 60 ml 2-methoxyethanol; pH 3.25	Sample loading, elution of aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine and cystine
3	18	8 g sodium hydroxide and 21 g citric acid monohydrate; pH 4.25	Elution of valine, methionine, isoleucine, leucine and norleucine
4	30	6.4 g sodium hydroxide, 12.6 g citric acid monohydrate and 15.25 g borax ($Na_2B_4O_7 \cdot H_2O$); pH 7.00	Elution of tyrosine, phenylalanine, anisylalanine and histidine
5	22	4 g sodium hydroxide, 10.5 g citric acid monohydrate and 19 g borax pH; 10.00	Elution of tryptophan, lysine, ammonia and arginine
6	4	0.2 *M* sodium hydroxide	Column regeneration

* Composition per litre; all contained 1 ml thiodiglycol, 1 g Brij 35 and 0.1 ml of sodium pentachlorophenol solution (1 g/100 ml ethanol). NaOH or HCl was added to adjust to correct pH.

colorimeter channel were observed (Fig. 1) and these were thought to be due to the alkaline column eluting buffer slightly increasing the pH of the reagent. The closer the reagent pH approached neutrality the greater was the increase in yellow colour which absorbed more at 440 nm than at 570 nm.

Buffers of high pH eluted the basic amino acids more rapidly than those of increased sodium molarity[4] and chromatograms produced by high molar buffers took 20–30 min longer to complete compared with those illustrated in Figs. 1 and 2, with a corresponding decrease in sensitivity as substances emerged as broader peaks.

Solutions of citrate and borate do not buffer well between pH 6.50 and 8.50 (ref. 1). This is of little consequence with protein hydrolysates but may prove a limiting factor in the analysis of the more complex amino acid mixtures of physiological fluids if stepwise buffer changes are employed. Gradient elution is superior for this purpose (Ersser *et al.*[5]).

ACKNOWLEDGEMENTS

The author is indebted to Mr. D. Stelling and members of his application staf at Rank-Hilger Ltd., Margate, Kent, for information concerning the suitability o borate buffers for amino acid chromatography. Mr. P. Winfield, Department o Biology, University of Southampton, provided the sample of hydrolysed bean mea illustrated in Fig. 1.

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