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STEP-CHANGE *versus* GRADIENT LITHIUM BUFFERS FOR SINGLE-COLUMN CHROMATOGRAPHY OF COMPLEX AMINO ACID MIXTURES*

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SUMMARY

Separation of more than fifty acidic, neutral, and basic amino acids, including asparagine and glutamine was achieved on a single 60 × 0.9 cm column of spherical resin using lithium citrate buffers. Usefully improved resolution results from a gradient *vs.* a step-change buffer system. Some limitations of the methods especially with complex natural samples having numerous unknowns and variable concentrations, are considered. Elution positions of additional less common amino acids are given.

INTRODUCTION

Since BENSON *et al.*[1] introduced lithium citrate to separate asparagine and glutamine without sacrificing resolution of other common neutral and acidic amino acids, others[2–7] have reported similar successes using various modifications. PETERS *et al.*[2] used the same resin, *i.e.*, Beckman's Type PA-28; MONDINO[3] prepared crushed Amberlite IR-120H; and ATKIN AND FERDINAND[4] tried Bio-Rad's Aminex A-5. However, these investigators used step-change buffers only and did not analyze the basic amino acids on the same column.

For selected synthetic reference mixtures, these accelerated two- to four-column approaches appear adequate. For complex natural materials (often of precious, limited sample supply and containing multiple unknowns and widely varying concentrations) there are interferences and limitations that put the analyst squarely in the judgement dilemma, described so well by HAMILTON[5], of either having to compromise resolution in favor of gaining time or *vice versa*. ATKIN AND FERDINAND[4] attempted to handle this problem by a scheme of juggling pH, Li^+ concentration, and especially *n*-propanol content of their buffers. Unfortunately, four columns, multiple sample aliquots, very careful standardization after each manipulation of an operating variable, and replicate runs appear essential to resolve less common ninhydrin-positive compounds and achieve necessary credibility in identifications based on elution time.

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Even then, there are problems of unsatisfactory separation of the amides, and of inevitable crowding with attendant uncertainties for common compounds immediately following second-buffer breakthrough.

PERRY *et al.*[6], and VEGA AND NUNN[7] sought to minimize such handicaps by using single 0.6 × 130-cm or longer columns and continuous gradient buffers. Physical capacity of their apparatus limited flow-rates to 30 ml/h, but this slow rate together with double-length columns provided improved resolution in 20–24 h runs. Column resins were Technicon's chromobeads, Type B and Type A, respectively.

This paper reports concurrent work with another spherical bead resin that: (a) confirms, yet qualifies the advantage of lithium over sodium in resolving asparagine and glutamine from some of the common amino acids; (b) compares a gradient *vs.* step-change lithium buffer system for a 0.9 × 60-cm single-column assay of more than sixty components yielding usefully improved resolution in the critical second buffer break-through area; and (c) emphasizes some limitations of the methods encountered in assay of "free" amino acids from plant and soil extracts. For many other owners wedded by large investment and long experience to older analyzers, the report may be of special interest.

MATERIALS AND METHODS

Reagents

Buffers. The composition of the lithium citrate buffers used for the two systems is shown in Tables I and II. Guidance for selection of high concentration and pH for the third buffer came from HAMILTON's classic paper[8].

Ninhydrin. The ninhydrin reagent mixture was prepared according to the method of SPACKMAN *et al.*[9].

Lithium hydroxide. A solution of 0.3 *N* lithium hydroxide was used to regenerate the resin after analysis.

Equipment. The amino acid analyzer was a Phoenix Precision Instrument Company (PPI) Model K-8000A designed for automated step-change (rather than

TABLE I

LITHIUM CITRATE BUFFERS FOR STEP-CHANGE SYSTEM

Switch from buffer A to B at 220 min; buffer B to C at 345 min; column temperature was changed from 35 to 60° at 480 min.

Item	*Buffer*		
	Li-A	*Li-B*	*Li-C*
pH	2.80±0.01	4.10±0.01	4.90±0.01
Lithium concentration, *N*	0.3	0.3	0.8
Components, per liter:			
Lithium citrate·4H_2O, g	28.2	28.2	28.2
Lithium chloride, g	—	—	21.2
HCl (conc.), ml	19.5	12.0	4.0
Thiodiglycol (25%), ml	20	20	—
Na_3 EDTA, g	1.0	1.0	—
Pentachlorophenol (5 mg/ml ethanol), ml	0.1	0.1	0.1

TABLE II

LITHIUM CITRATE BUFFERS FOR GRADIENT SYSTEM

A column temperature change from 35 to 60° was initiated at 180 min.

Item	*Buffer*		
	Li-I	*Li-II*	*Li-III*
pH	2.63±0.01	3.63±0.01	6.15±0.01
Lithium concentration, *N*	0.2	0.2	1.2
Components, per liter:			
Lithium citrate·$4H_2O$, g	18.8	18.8	18.8
Lithium chloride, g	—	—	42.4
HCl (conc.), ml	13.0	8.8	0.2
Thiodiglycol (25%), ml	20	20	—
Na_3 EDTA, g	1.0	1.0	—
Pentachlorophenol (5 mg/ml ethanol), ml	0.1	0.1	0.1
Autograd:			
Buffer, ml/chamber	100	100	100
Chamber numbers	1–3	4–5	6–9

gradient) buffer program as devised by SPACKMAN *et al.*[9]. State-of-the-art improvements added through the years have improved resolution and sensitivity to the equivalent of current PPI K-8000C or Beckman-Spinco 120C analyzers. (Some of the more sophisticated valving, autoloading, and programming options would give additional flexibility but, for non-routine research samples, are currently difficult to justify.) More important modifications permitting accelerated, high-resolution operation included: high-pressure columns, fittings, and gauges to >600 p.s.i.; spherical bead PPI Spherix XX8-60-1 resin — normally a 60-cm bed in a 0.9 × 69-cm column; 4-mm path-length flowcell (physical limit of the original photometer); sapphire plungers in buffer and ninhydrin pumps; in-line, Teflon-sponge, buffer filters[10]; programmed microvalves for automated column regeneration; and a multiple-speed, dual-range recorder — 2 or 10 mV full scale by simple change of a resistor card. Without extending the Teflon reaction coil length or reaction time, and at a normal flow-rate of 60 ml/h, 50-n*M* reference standards provide one-half to full scale deflection on the 2 mV recorder range. Further optical or electronic amplification for sub-nanomole sensitivity demands such extensive precautions to eliminate spurious background and avoid inadvertent contamination (HAMILTON[5,11]) that the costs can become prohibitive. Greater sensitivity with less risk of misidentification and erroneous quantitation is, of course, much easier when one can couple ninhydrin and radioactivity assay — the latter most conveniently by a continuous liquid-flow scintillation detector[12].

Procedure. Nominal operation included the following: 5–500 n*M* amino acid concentration; 60 ml/h buffer and 25 ml/h ninhydrin flow-rates; compromise 4 in./h chart with 1 dot/2 sec print speed for adequate peak area yet reduced chart bulk; 35 and 60° column temperature program; three buffers each for step-change or gradient elution program. Tables I and II summarize buffer composition, pH, volumes, and program. A nine-chamber Autograd (Technicon) was used for continuous gradient in place of step-change buffer elution when appropriate.

RESULTS AND DISCUSSION

Fig. 1 shows the comparative elution positions of more than fifty compounds chromatographed with step-change and gradient lithium buffers in $15^1/_2$ h on a single 0.9 × 60-cm column. Good separation of the more common amino acids was achieved except for undesirable crowding in the cystine-to-leucine area with step-change buffer. Both systems resolved asparagine, glutamic acid, and glutamine. α-Aminoadipic acid precedes proline, and citrulline follows alanine; this agrees with PETERS *et al.*[2], and contrasts with BENSON *et al.*[1], and PERRY *et al.*[6]. Neither system separates homoserine from glutamine, but each separates homocystine and most of the basic compounds.

Elution of most compounds was slower with the gradient buffer because of lower pH and Li^+ concentration in the first two buffers; cystine and tryptophan were exceptions. The amino sugars were most sharply delayed. Broad-peaked galactosa-

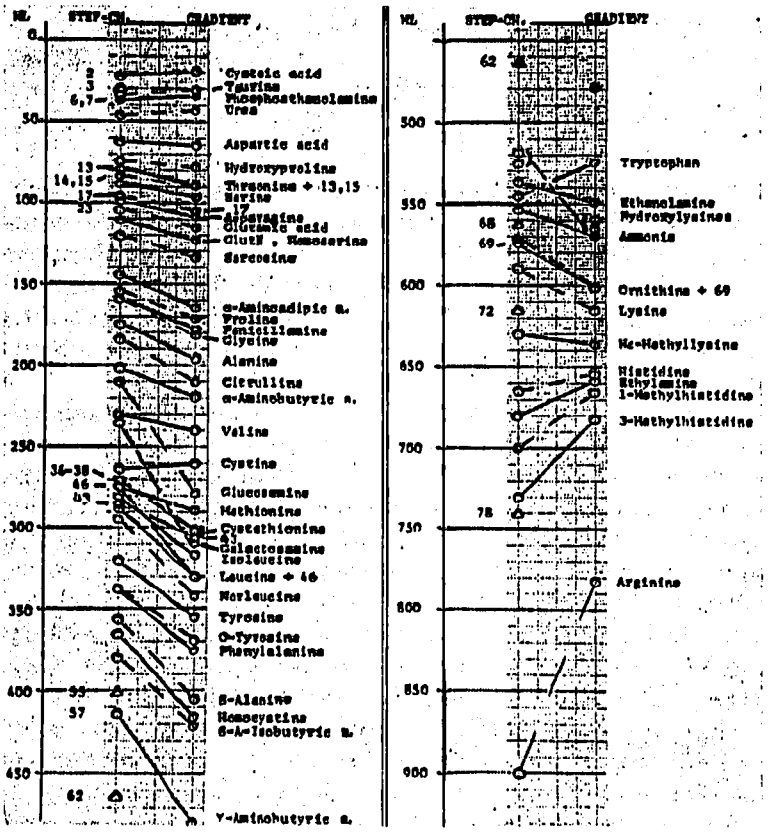

Fig. 1. Comparative elution time of amino acids and related compounds. Step-change *versus* gradient buffer; flow-rate, 60 ml/h. Abbreviations used in nomenclature: a. = Acid; A- = amino. Compounds indicated by numerals: 2 = phosphoserine, 3 = allantoic acid, 6 = levulinic acid, 7 = allantoin, 13 = methionine sulfone, 14 = threonine, 15 = methionine sulfoxide, 17 = α-methylserine, 23 = α-methylglutamic acid, 36 = pipecolic acid, 37 = norvaline, 38 = arginosuccinic acid, 43 = 3,4-dihydroxyphenylalanine, 46 = α,ε-diaminopimelic acid, 55 = δ-aminolevulinic acid, 57 = α-aminooctanoic acid, 62 = δ-aminovaleric acid, 68 = ε-aminocaproic acid, 69 = 2,4-diaminobutyric acid, 72 = methylamine, 78 = canavanine.

mine shifted by more than 1 h, from the trailing edge of valine to overlap with double-peaked cystathionine. These differential movements resulting from changed operating conditions may unsuspectingly alter the sequence of elution for neighboring compounds (*e.g.* methionine, diaminopimelic acid, hydroxylysines, ammonia, ethanolamine, tryptophan). Such behavior gives clear but often neglected warning against simple reliance on sequence charts reported by others, even though such charts may be very helpful.

Elution positions of many less common ninhydrin-positive compounds were determined because the variety of non-routine research samples from soil, water, plant, exudate, microbial, and cellular materials encompasses such a range of concentrations and multiple unknowns that necessity requires a sacrifice in speed of analysis in favor of resolution.

Procedure variables

Initial column temperature. A higher temperature in both systems hastened elution of glutamic acid more than asparagine and glutamine, hence separation between asparagine and glutamic acid was worse but that between glutamic acid and glutamine was improved. A higher temperature also hastened α-aminoadipic acid more than proline, thus improving their separation.

pH of Li-A and Li-I buffers. A higher (less acid) pH of the initial buffer had the same effect as higher initial temperature.

pH of Li-II buffer. A higher pH of Li-II had a similar effect to higher temperature on the separation of α-aminoadipic acid and proline. Higher pH also hastened citrulline more than alanine and α-aminobutyric acid, thus separation between alanine and citrulline lessened, while that between citrulline and α-aminobutyric acid improved.

Column resin. Subtle differences between resins and apparatus sometimes appear inordinately crucial to adequate resolution, especially with step-change buffers where components are closely bunched in certain areas of the chromatogram. For example, Spinco's UR-30 resin, which offered lower back-pressures and worked well for Beckman people, failed in our unit. Temperature and pH factors were critical. Adjustments in pH of $< \pm 0.01$, or changes in temperature of $< \pm 1°$ to resolve the asparagine–glutamic acid–glutamine group disrupted resolution of the proline–glycine pair, cystathionine–methionine pair, or both. MONDINO[3] also found pH and temperature very critical using lithium buffer on crushed Amberlite IR-120H, as did ATKIN AND FERDINAND[4] on Aminex A-5. LONG AND GEIGER[13] systematically examined effects of small differences in resins.

Of several other resins tried, including Aminex A-7, the Spherix XX-8-60-1 gave the best performance overall in our Phoenix equipment. As with the other resins, however, serious overcrowding remained in the cystine-to-norleucine region. Lowering the pH of the second buffer from a recommended 4.15 (BENSON *et al.*[1]) to 3.85 confirmed the improvement cited by PETERS *et al.*[2] except that cystine was still confounded by buffer breakthrough; also, later peaks were undesirably broadened. A 15-min, buffer switching delay to clear cystine caused cystathionine overlap into methionine. Worse still, the second buffer of pH 3.85 caused a shift of several basic amino acid components to mutually interfering positions. Adjustments of buffer C through the range pH 4.50 to 5.28 at 0.8 *M* Li^+, showed 4.90 to be best in combination with buffer B at 4.10. Higher pH, stronger ionic strength, and earlier temperature rise (all to speed arginine

elution) caused unacceptable overruns of important compounds such as ethanolamine, ammonia, lysine, tryptophan, ornithine, and the histidines.

Limitations with unknowns

With synthetic reference mixtures, avoiding uncertainties of interpretation is relatively easy. However, "judicious" selection of reference components and concentrations easily creates a false sense of security. The hazard is compounded readily by the common tendency (a) to disregard low concentration or less common substances, and (b) to exclude from the reference mixture most components that show poor resolution on the elution diagram.

The uncertainties and risks of misidentification are considerably greater with natural samples having multiple unknowns and widely varying concentrations. This bears emphasizing with some illustrations of limitations we encountered in assaying "free" amino acids from soil and plant extracts using step-change lithium buffers. (a) Unless samples are "cleaned up" by a series of extraction and desalting operations, a clutter of pigments, organic acids, and numerous ninhydrin-positive compounds elute early. Commonly, qualitative identifications, let alone quantitation, of components before aspartic acid are uncertain from the ninhydrin pattern alone. Also, other less mobile ninhydrin-negative compounds may interfere with normal migration or color development of subsequent amino acids[14]. On the other hand, "clean-up" usually results in unavoidable partial losses of many constituents; these losses are uncorrectable without a great amount of costly analytical work for which sufficient sample or justification is often lacking. (b) The sulfoxides of methionine overlap threonine. Fortunately, "free" methionine levels are relatively very low in many plant and soil extracts, thus precluding significant error from that source in quantitation of threonine. For hydrolysates of high sulfur content proteins, such interference must, of course, be considered. (c) Homoserine elutes exactly with glutamine. Homoserine appears in numbers of plant and soil extracts (most notably from peas, *Pisum sativum* L.), and, by reason of such masking by glutamine, may be more common than generally believed. (d) The amino sugars glucosamine and galactosamine, if present in appreciable concentration (not uncommon for glucosamine), can seriously overlap valine, particularly if the pH of the first buffer is slightly off. (e) Cystine appears as a very sharp peak with breakthrough of the second buffer. But, variable amounts of unknowns usually also elute with buffer breakthrough, thus preventing reliable identification or quantitation of cystine. (f) Crowding and overlap of other components in the cystathionine, methionine, isoleucine, leucine region cause qualitative and quantitative uncertainty for numbers of samples. In soil extracts, for instance, α,ε-diaminopimelic acid (of microbial origin) is of interest; this elutes with and confounds methionine. A different example for plant extracts is overlap of pipecolic acid and cystathionine[12]. For such cases, automated read-out based on elution time is erroneous. We averted serious misidentification as cystathionine only by reason of a coincident ^{14}C-labeled peak ultimately traced to pipecolic acid as a metabolite from labeled lysine (Fig. 1 in ref. 12). (g) Other very close pairs that permitted easy misidentification, when only a ninhydrin pattern was used, were α-aminooctanoic acid *vs.* γ-aminobutyric acid, and ε-aminocaproic acid *vs.* ammonia[12]. Amounts of ammonia are often so large relative to other components in the neighboring cluster, from ethanolamine to ornithine, that reliable identification or quantitation is precluded.

Plainly, the lithium-buffer, single-column approach with step-change analyzers has important advantages, but for non-routine complex research samples it is not a panacea. A gradient rather than step-change buffer program gives fewer overlaps and better spread of peaks in the same running time. Of course, with progressively longer columns and slower flow-rates, resolution can be improved[6,7], and in skilled hands, to unprecedented limits[5]. Nevertheless, compromise is virtually inevitable. Unfortunately, as HAMILTON indicates, too often in the analysis of complex biological mixtures, "...resolution is compromised at the outset in favor of speed of analysis..." The resolution of components in urine samples as achieved by KEDENBURG's recent adaptation[15] contrasted to that achieved by HAMILTON[5] is illustrative. Where the overlap and crowding of important components following buffer breakthrough can be tolerated, KEDENBURG's two-lithium-buffer, single-column 8- to 10-h system (allowing for regeneration/re-equilibration and sample loading time) offers potential economies from two rather than one assay per day. But, in dealing with complex mixtures from biological research samples wherein multiple unknowns and trace quantities are more the rule than the exception, some sacrifice of time in favor of resolution appears the more desirable compromise. There appears to be a need to stem the flood of questionable chromatographic (and other) data issuing from sophisticated, though rather easily operated automated analyzer, data-reducer, and computer systems, sometimes simply because they are available. This could be largely accomplished and the corrosive dilemma of having to choose between resolution and speed could be minimized simultaneously by less subconscious acquiescence to pressures about "how fast and how much data can we own," and by more consciously questioning "will the data be worth owning?"

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