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Amino Acid Analysis by Reverse-Phase High-Performance Liquid Chromatography: Precolumn Derivatization with Phenylisothiocyanate[1,2]

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Methods for the quantitative derivatization of amino acids with phenylisothiocyanate and for the separation and quantitation of the resulting phenylthiocarbamyl derivatives by reverse-phase high-performance liquid chromatography are described. Phenylthiocarbamylation of amino acids proceeds smoothly in 5 to 10 min at room temperature. Coupling solvents, reagent, and some byproducts are removed by rotary evaporation under high vacuum, and the phenylthiocarbamyl derivatives are dissolved in 0.05 M ammonium acetate, pH 6.8, for injection onto the octyl or octadecylsilyl reverse-phase column. Columns are equilibrated with the same solvent and the effluent stream is monitored continuously at 254 nm for detection of the amino acid derivatives. Elution of all of the phenylthiocarbamyl amino acids is achieved in about 30 min utilizing gradients of increasing concentrations of ammonium acetate and acetonitrile or methanol. This approach to amino acid analysis offers select advantages, both with respect to methods which employ reverse-phase separation of prederivatized samples and to the classical ion-exchance procedure. All amino acids, including proline, are converted quantitatively to phenylthiocarbamyl compounds and these are stable enough to eliminate any need for in-line derivatization. Furthermore, results comparable in sensitivity and precision to those obtained by state-of-the-art ion-exchange analyzers may be generated with equipment that need not be dedicated to a single application.

This issue is dedicated to the memory of Stanford Moore, who, with his associate, William Stein, working together for nearly four decades, helped lay the foundation of the field of protein chemistry as we know it today. One of their crowning scientific achievements was the development of an ion-exchange chromatographic procedure for quantitative amino acid analysis. The application of chromatographic methods in the 1940s revolutionized the biological sciences and separation chemistry in general, for it was now possible for the first time to resolve the complex mixtures encountered in living systems; proteins and nucleic acids became targets for chemical characterization.

Moore and Stein were acutely aware of the potential of chromatography and of the need for a method for quantitative amino acid analysis as a first step towards the derivation of complete covalent structures of proteins. Their early efforts in the mid-1940s to resolve mixtures of amino acids by partition chromatography on starch columns soon gave way to the use of the then newly developed ion-exchange resins. In 1958 two papers from their laboratory appeared which described the ion-exchange chromatographic separation of all of the amino acids expected from protein hydrolysis on columns of sulfonated polystryene and which presented details for construction of an automated analyzer (1,2). In these classic papers, ninhydrin was established as the means for postcolumn derivatization of the amino

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acids for purposes of accurate spectrophotometric quantitation. Although subsequent refinements in columns and instrumentation have reduced the time of analysis from days to minutes and the sensitivity from micromoles to picomoles, this procedure remains essentially unchanged in 1983, and is still the most widely employed means for quantitative amino acid analysis. In fact, ion-exchange separation of amino acids sets the standard of excellence by which any method which might be proposed as an alternative must be judged.

In the 1970s a revolution in analytical chemistry was brought about by the development of reverse-phase separation procedures and the advent of high-performance liquid chromatography. Of course, current ion-exchange analyzers are really HPLC systems, but the new stationary phases of greatest interest were those reverse-phase adsorbants of silica modified with octadecyl, octyl, or other organic adducts. The purification of peptides and the separation of phenylthiohydantoin (PTH)[3] amino acids on columns of this kind has greatly facilitated the task of protein sequencing (cf. (3)), and it seemed highly probable that reverse-phase HPLC would have an equal impact in the area of amino acid analysis. Accordingly, if amino acids could be converted quantitatively to a mixture of derivatives amenable to separation by reverse-phase HPLC, and having spectral characteristics consistent with detection at the picomole level, one could devise a very simple, sensitive, and precise amino acid analyzer.

Attempts toward this end have been made, based primarily upon precolumn modification with reagents which give fluorescent amino acid derivatives. The use of *o*-phthalaldehyde (4–7) offers high sensitivity capabilities but suffers from disadvantages relative to the lack of proline reaction, instability of the fluorescent products, and difficulties in quantitation due to the sensitivity to quenchers. Quantitation of proline and hydroxyproline requires special oxidative procedures (8) for opening the ring in these amino acids thus greatly complicating the design of *o*-phthalaldehyde-based analyzers. Dansyl chloride reacts with proline, but this often employed end-group reagent is more sluggish in reactivity and, although its use in conjunction with amino acid analysis has been described in several papers (9–13), the method has not gained wide acceptance. Another precolumn derivatization system employing dimethylaminoazobenzene-4′-sulfonyl chloride has been described by Chang *et al.* (14,15), but, like dansylation, arylation with these sulfonyl halides presents difficulties in quantitative modification, and application of this approach has been greatly limited.

We sought to develop a method for the derivatization of amino acids and for the separation of these derivatives by reverse-phase HPLC, optimized according to the following criteria: the method should be quantitative, simple, rapid, sensitive, and reproducible. A reagent was sought which would yield derivatives detectable at picomolar concentrations by uv absorbance. Detection in the uv range would have the advantage of providing a simple relationship between the concentration of the amino acid derivative and the electrical signal of the detector and would thus circumvent the shortcomings in quantitation of fluorescent compounds. The derivatization step should proceed rapidly and quantitatively with all amino acids and the derivatives should have roughly equal extinction coefficients. The method of derivatization should itself be simple and, ideally, reagent, byproducts, and solvents ought to be volatile and easily removable by evaporation. At the same time, however, a simple method for routine laboratory use should not be excessively fussy, and should yield the desired results even within a wide error tolerance of performance of the technique.

This communication describes the use of the Edman reagent, phenylisothiocyanate

[3] Abbreviations used: PITC, phenylisothiocyanate; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin; CMCys, carboxymethylcysteine; RCM, reduced and carboxymethylated; HOPro, hydroxyproline.