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THE USE OF *o*-PHTHALALDEHYDE FOR FLUORESCENCE DETECTION IN CONVENTIONAL AMINO ACID ANALYZERS

SUB-NANOMOLE SENSITIVITY IN THE ANALYSIS OF PHENYLTHIOHYDANTOIN–AMINO ACIDS

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SUMMARY

A conventional amino acid analyzer has been modified for fluorescence detection using *o*-phthalaldehyde. The original ninhydrin system is retained, and the amino acid analyzer can be used with either of the detection systems. The fluorescence detection is used for identification of PTH–amino acids after back hydrolysis, when nanomole amounts of proteins are sequenced. Impurities in the water and the chemicals used for buffer preparations are limiting factors for the sensitivity of the fluorescence detection system. A 100-fold increase in sensitivity above the original ninhydrin system is obtained.

INTRODUCTION

Recently there has been increased interest in the sequence analysis of proteins accessible only in small amounts. Accordingly, procedures have been developed for the automatic sequencing of nanomole amounts of proteins and for the detection of sub-nanomole amounts of phenylthiohydantoin (PTH)–amino acids. By the use of gas chromatography[1], supplemented by thin-layer chromatography on polyamide sheets[2], most of the PTH–amino acids may be readily identified. Identification of PTH–arginine and –histidine, and differentiation between PTH–leucine and –isoleucine, are, however, often more reliable when supported by amino acid analysis after hydrolysis of the PTH–amino acid, *i.e.* back hydrolysis. The need for greater sensitivity led to an investigation of the use of *o*-phthalaldehyde[3] as a reagent in a conventional amino acid analyzer employing columns of 9 mm bore.

Systems designed for the fluorimetric determination of amino acids at picomole levels have been available for 2–3 years, utilizing fluorescamine. A greater sensitivity compared with ninhydrin was not, however, obtained in amino acid analyzers with microbore columns, as demonstrated by Benson and Hare[4]. Fluorescamine is usually dissolved in acetone since it hydrolyzes in water, and a buffer

solution must therefore be added to the column effluent, requiring an additional pump. It would not be economical at present to use fluorescamine in amino acid analyzers with columns of large bore.

EXPERIMENTAL

Apparatus

A Beckman Model 120C amino acid analyzer, updated with an automatic sample injector Model 121, was used for the chromatography. The fluorescence was measured in a Perkin-Elmer Model 1000 spectrofluorimeter and recorded on a Honeywell single-channel potentiometric recorder. The wavelengths used were 340 nm for excitation and 455 nm for emission.

Reagents

Buffered reagent. *o*-Phthalaldehyde was purchased from Hoechst, Sigma or E. Merck. A 0.8 *M* potassium borate buffer was prepared from a suspension of boric acid which was titrated with potassium hydroxide to pH 9.7. 4 ml of 2-mercapto-ethanol were added to 4 l of borate buffer, followed by 3.2 g of *o*-phthalaldehyde dissolved in 25 ml of 96% ethanol. Finally, 12 g of Brij (30%) was added to the solution.

Citrate buffers. Sodium citrate (Merck No. 6430, for amino acid analysis), hydrochloric acid [E. Merck, Darmstadt, G.F.R.; analytical grade, or 30% (Suprapur)], and twice-deionized water were used for the buffer at pH 5.25. The buffers at pH 3.25 and 4.25 were prepared from sodium citrate (Fluka, Buchs, Switzerland; puriss. p.a.), analytical-grade hydrochloric acid distilled over sodium dichromate[5] and twice-distilled water, the second distillation being over potassium permanganate. The buffers were prepared immediately before use and filtered through a 0.22-μm membrane filter. No anti-mould or anti-oxidant reagents were added.

In recent experiments the buffer change peak (see Fig. 3) was significantly reduced due to the use of water purified by the Milli-Q system produced by Millipore Corporation.

RESULTS AND DISCUSSION

In the system described, the only modification of the amino acid analyzer is the insertion of one of the existing three-way slide valves into the effluent to coil line leading from the four-way motor valve. An extra pump and tee, earlier used for stream division, is used for delivery and mixing of the fluorigenic reagent with column effluent, see Fig. 1. The original ninhydrin system is thus retained, and the analyzer can therefore be used with either detection system. The buffer flow-rate was 70 ml/h and the reagent flow-rate was 35 ml/h.

The mixture of the reagent and the column effluent was connected to the flow cell in the fluorimeter by means of PTFE tubing (1 m × 0.3 mm I.D.). Since no suitable commercial flow cell was available, a flow cell was blown from heavy-walled glass tubing (5 mm O.D.), see Fig. 2 and centered in the standard cuvette holder by use of two pieces of rubber tubing. By shielding the cuvette holder with black adhesive tape, the influence of stray light was avoided. The optimal inner diameter of the cell

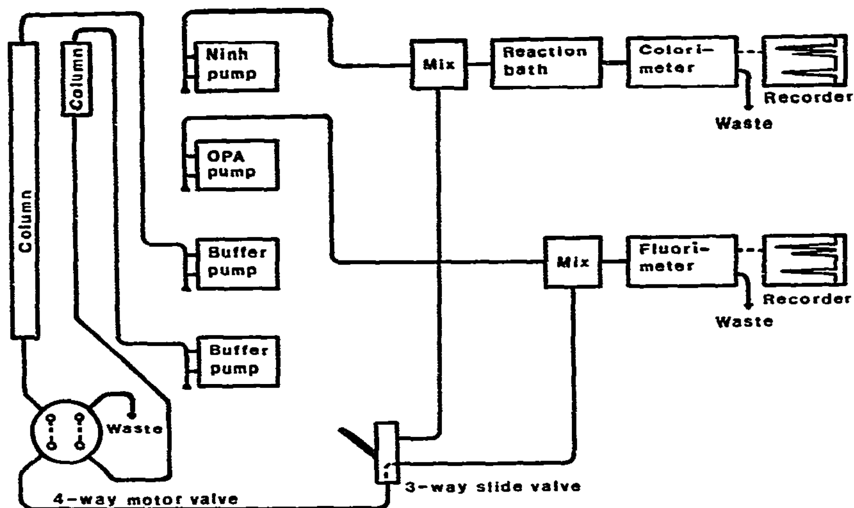

Fig. 1. Flow diagram of a conventional amino acid analyzer modified for fluorescence detection. OPA = *o*-phthalaldehyde reagent; Ninh = ninhydrin reagent.

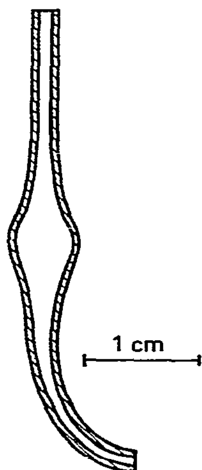

Fig. 2. Flow cell used in the spectrofluorimeter.

was *ca.* 4 mm. In order to avoid unnecessary mixing in the flow cell, the volume was kept as small as possible and the angles were made smooth. The signal-to-noise ratio is dependent on the shape of the cell and on the position and shielding. However, these parameters are easily optimized.

The system was developed in order to allow identification of PTH–amino acids in sub-nanomole amounts after back hydrolysis, see Fig. 3A. Its use can be extended to quantitative amino acic analysis except for proline and hydroxyproline. As shown in the chromatograms in Fig. 3, the lower limit of the sensitivity is determined by the difference in baseline levels and by the peak caused by changing the buffer on the column for the acidic and neutral amino acids. A twenty-fold increase in sensitivity

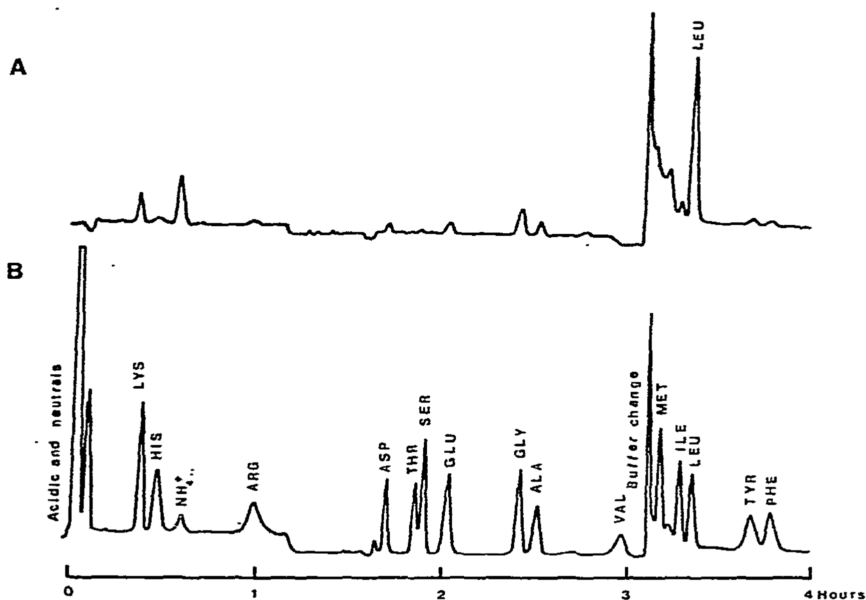

Fig. 3. Chromatograms showing the identification of leucine after back hydrolysis of the PTH derivative from the sequencer (A), and a calibration mixture (Pierce Type H) containing 0.5 nmole of each amino acid (B). Regarding reduction of the buffer change peak, see under *Citrate buffers*.

is still available on the fluorimeter, compared to the sensitivity used at present. Impurities in the distilled water and the chemicals used for the buffer preparations seem to be limiting factors for a further increase in sensitivity. Chemicals from different manufacturers were tested and considerable differences were found in the contents of contaminants. We believe that it is possible to achieve a sensitivity of 0.5 nmole of each amino acid for full-scale deflection with commercial reagents. Higher sensitivities would be very inconvenient to administer, due to difficulties in the sample preparation, proper cleaning of glassware and contamination from the sample injection system.

The system described may be used in any conventional amino acid analyzer. The use of *o*-phthalaldehyde is also economical with the large columns of 9-mm bore. Since the buffer: reagent flow ratio is the same as in the ninhydrin system, the ninhydrin pump may be used for delivery of the *o*-phthalaldehyde reagent without flow adjustment in the case of amino acid analyzers which are not equipped with an additional pump. In comparison with the sensitivity of a colorimeter with selenium photocells, a 100-fold increase in sensitivity is readily obtained.

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