Journal of Chromatography, 175 (1979) 183–188

CHROM. 11,875

Note

Simultaneous determination of radioactivity and o-phthalaldehyde derived fluorescence in low levels of amino acids

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(Received February 14th, 1979)

Accurate estimation of protein turnover, *in situ*, requires correction for re-utilization[1] by measuring the specific radioactivity of the labeled amino acid(s) in the protein precursor pool, *i.e.* amino acids acylated to tRNA. One method of determining specific radioactivity of amino acids involves dividing the column effluent of an amino acid analyzer prior to color development so that radioactivity can be determined on a portion of the column effluent directly. Stream splitting results in a reduction of sensitivity, yet is necessary to eliminate color quenching and, when ^{14}C is measured, loss of radioactivity in the form of $^{14}CO_2$ as a result of the ninhydrin reaction[2].

The development of *o*-phthalaldehyde[3] as a reagent for quantitating primary amines has increased the sensitivity of amino acid analyses from μmole to pmole amounts[4,5]. Although the mechanism of reaction between *o*-phthalaldehyde and primary amines is unknown[3], quantitative recovery of amino acid carbons in the fluorophore would allow determination of radioactivity in the spectrofluometer effluent directly. Amino acid analyzers, equipped with microbore columns and detection systems using *o*-phthalaldehyde, that are capable of quantitating α-amino acids in the pmole range are either commercially available or have been described[4,6]. Many laboratories, however, have conventional amino acid analyzers (6- or 9-mm diameter column); modification of those instruments for quantitation of amino acids using *o*-phthalaldehyde would greatly expand research capabilities when specific radioactivity of low levels of amino acids is desired. This note describes the simple modification of a conventional amino acid analyzer to attain pmole sensitivity and demonstrates the utility of measuring radioactivity in the spectrofluorometer effluent.

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MATERIALS AND METHODS*

Equipment modifications

A Beckman 119B automatic amino acid analyzer complete with two ninhydrin reservoirs and three Accu-Flo pumps was outfitted with a 60 × 0.6 cm glass column (Glenco Scientific, Houston, Texas, U.S.A.). The column was packed with Durrum DC-6A cation-exchange resin (Durrum, Palo Alto, Calif., U.S.A.,) to a height of 37 cm.

PTFE tubing and standard 1/4 × 28 flare fittings (Durrum) were used to make liquid connections in the fluorometric modification of the analyzer. One ninhydrin reservoir and Accu-Flo pump was used to store and pump the fluorometric reagent. The outlet of the pump was connected, with 0.5-mm I.D. tubing, to one port of a 3-port manifold (0.8-mm bore), which served as a mixer. A 5-cm segment of 0.5-mm I.D. tubing connected the outlet of the 6-mm column to a second port of the mixing manifold. Thin-wall PTFE spaghetti tubing (28 AWG; Read Plastics, Rockville, Md., U.S.A.), 170 cm, fitted with microbore tubing adapters was used to connect the outlet of the mixing manifold to the fluorometer flowcell; a 70-cm segment was immersed in a water-bath maintained at 35° (Haake, Saddlebrook, N.J., U.S.A.).

A spectrofluorometer (Model 430, G. K. Turner Assoc., Palo Alto, Calif., U.S.A.) was equipped with a 70-μl round flowcell (American Instrument Co., Silver Spring, Md., U.S.A.) by mounting the flowcell and its holder in a 13 mm^2 × 5 cm long aluminium block machined with appropriate slits and positioning screws**. The flowcell was maintained at 35°. Excitation wavelength of the spectrofluorometer was set at 350 nm; emission wavelength at 450 nm. Effluent from the spectrofluorometer flowcell was directed to a fraction collector set to collect fractions at 3-min intervals. Output from the spectrofluorometer was displayed on a strip-chart recorder.

Reagents and procedures

Sodium buffers (Beckman, Fullerton, Calif., U.S.A.), pumped at the rate of 35 ml/h, were used as eluents. The fluorometric reagent, pumped at the rate of 17.5 ml/h, consisted of 0.63 *M* boric acid, 6 m*M* *o*-phthalaldehyde (*o*-phthalic dicarboxaldehyde; Aldrich, Milwaukee, Wisc., U.S.A. dissolved in a small volume of methanol) and 64 m*M* 2-mercaptoethanol, adjusted to a final pH of 10.4. Amino acid standards were from Beckman, and [1-^{14}C]leucine (57 mCi/mmole) from Schwarz-Mann (Orangeburg, N.Y., U.S.A.).

Radioactivity was determined by combining 2 ml of each fraction with 10 ml of liquid scintillation fluid (2 l toluene, 1 l Triton X-100, 16.5 g 2,5-diphenyloxazole) and subsequently counting in a Beckman LS 233 liquid scintillation counter. The quench of each sample was corrected by the external standards method. When the effluent and liquid scintillation fluid were mixed, a cloudy solution resulted which cleared within a few hours. A small phase separation occurred in some of the counting

* Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

** An adapter is offered by American Instrument Co. that allows the use of their flowcells in spectrofluometers equipped with standard 13-mm^2 cell holders.

vials, however when [^{14}C]leucine was added to these vials and the resulting counts corrected for quench, 100% of the added radioactivity was recovered.

Biological experiments

In an investigation of the labelling of the amino acids in muscle proteins, [1-^{14}C]leucine (15 μCi/100 g body wt.) was injected into the tail veins of 350–400 g male rats. After 30 min, the rats were sacrificed and the gastrocnemius muscle was immediately excised. Total muscle protein was precipitated by homogenizing muscles in 4% (w/v) sulfosalicylic acid. Aliquots of the precipitated protein were hydrolyzed with 6 *M* hydrochloric acid and the specific radioactivity of leucine was determined.

RESULTS AND DISCUSSION

A complete elution profile of the 16 common α-amino acids is shown in Fig. 1. One nanomole of each component and a standard three buffer program were used to generate the chromatogram (Fig. 1). The fluorometric response can be greatly increased for cystine by oxidation to cysteic acid prior to chromatography[7], and for lysine by addition of Brij to the *o*-phthalaldehyde reagent[4]. A buffer change peak and two unidentified peaks, which may be due to reagent impurities[7], are also evident on the chromatogram (Fig. 1). Although the sensitivity of the spectrofluorometer* can be increased several-fold, the extraneous peaks and baseline shifts limit the sensitivity of a complete analysis to about 0.5 nmole.

Since our primary interest was in quantitating the levels of leucine acylated to tRNA as part of our research on protein turnover, a single buffer program was established (Beckman buffer B, 0.4 *M* Na^+, pH 4.12) to resolve methionine, isoleucine

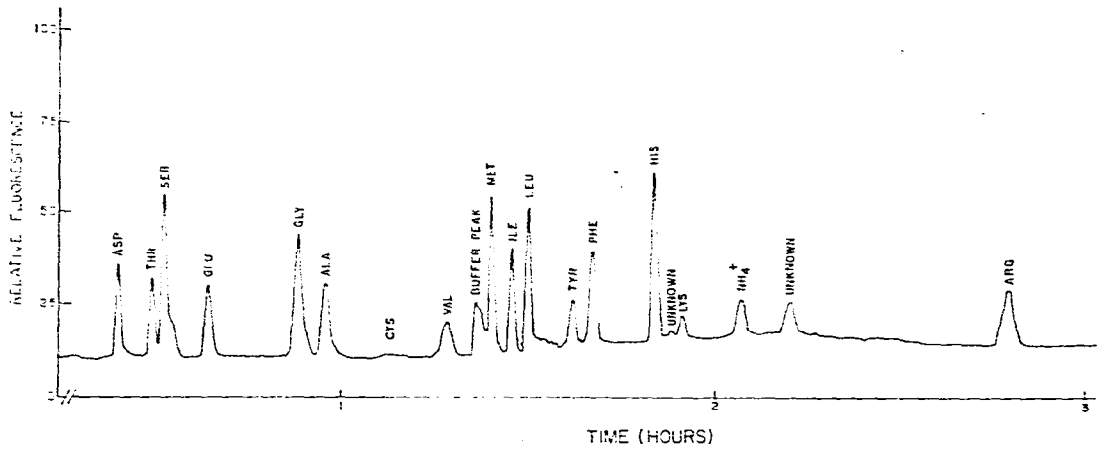

Fig. 1. Separation and fluorometric response of 16 α-amino acids (1 nmole each). A 37 × 0.6 cm column of DC-6A resin with a standard three-buffer (sodium citrate) program and a column temperature of 50° was used; the spectrofluorometer amplification was set at 30 to detect the *o*-phthalaldehyde derivative of each amino acid.

* A fluorometer (Turner, Model 111) and a fluoromonitor (American Instrument Co.) also were satisfactory as detectors in this system.

and leucine. The chromatogram shown in Fig. 2 represents a typical run with 75 pmoles of each amino acid. Using the single buffer program (buffer B, 20 min; sodium hydroxide, 10 min; reequilibration with buffer B, 25 min), the acidic amino acids were eluted early as a series of unresolved peaks followed by the well separated peaks of methionine, isoleucine and leucine. The remainder of the amino aicds were eluted from the column with sodium hydroxide as two large unresolved peaks. Sensitivity was greatly enhanced since the amino acids of interest were eluted with a single buffer thereby avoiding drastic baseline shifts and, as a result, the amplification of the spectrofluorometer could be increased to the maximum setting. Using the short program, peak area was linear with graded levels of leucine over the range of 0.025 to 10 nmoles.

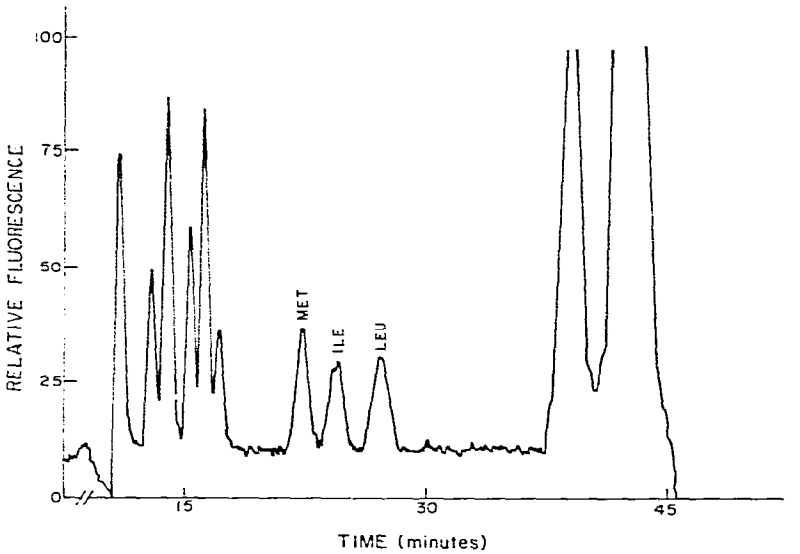

Fig. 2. Separation and fluorometric responce of methionine, isoleucine and leucine (75 pmoles each) during a high sensitivity run. A single buffer (buffer B) and temperature (50°) were used with the column described in Fig. 1; the spectrofluorometer amplification was set at 1000.

When [1-^{14}C]leucine was reacted with *o*-phthalaldehyde under the same conditions as the post-column chemistry of the analyzer, less than 0.1% of the total ^{14}C was recovered as $^{14}CO_2$. These results allowed us to determine the radioactivity in the spectrofluorometer effluent directly and to calculate the specific radioactivity of low levels of amino acids. The results of typical experiments are shown in Fig. 3. The separation of a standard amino acid mixture (250 nmoles of each amino acid) containing about 0.1 μCi of [1-^{14}C]leucine is shown in Fig. 3A and B. These data demonstrate that the radioactivity in the spectrofluorometer effluent was found in a single peak corresponding to leucine. Quantitation of the radioactivity in the spectrofluorometer effluent from several runs demonstrated that 93% of the radioactivity applied to the column as [^{14}C]leucine was recovered. Since about 2% of the radioactivity in [^{14}C]leucine is non-amino acid in nature[8], the net recovery of [^{14}C]leucine from the amino acid analyzer was 95%.

The fluorometric and radioactivity profiles of amino acids from muscle protein

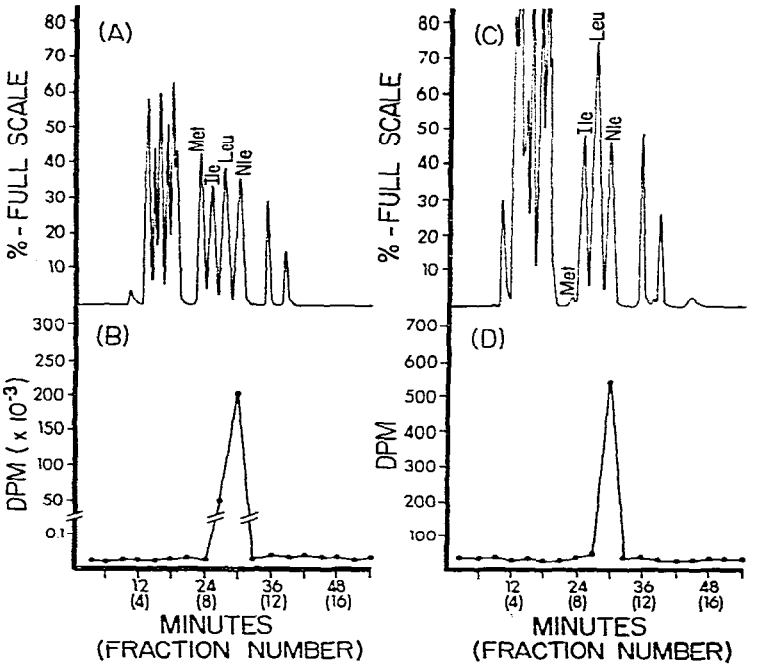

Fig. 3. Fluorometric and radioactivity profile of a standard amino acid mixture and a muscle protein hydrolyzate. (A) Fluorometric response of a standard amino acid mixture (250 nmoles of each amino acid) containing about 0.1 μCi of [1-^{14}C]leucine and eluted with a single buffer. (B) Fractions from (A) were collected and the radioactivity determined as described in Materials and methods. (C) Fluorometric response of a muscle protein hydrolyzate from a rat that had been injected with [1-^{14}C]-leucine; single buffer elution program. Internal standard, norleucine (Nle), was added to the sample immediately prior to analysis. (D) Fractions from (C) were collected and the radioactivity determined. The slight shift of the radioactive peak to the right (panels B and D) was caused by the delay of the solution in the tubing between the spectrofluorometer and the fraction collector.

hydrolyzates after injection of [1-^{14}C]leucine into the animal are shown in Fig. 3C and D, respectively. These observations demonstrate the feasibility of quantitating amino acids and the radioactivity associated with each amino acid in a biological sample on a modified amino acid analyzer. This system has been extensively used to determine the specific radioactivity of leucine in tRNA, free amino acids and proteins from rat muscle as well as blood plasma.

The modifications described in this note allow the specific radioactivity of low levels of amino acids to be determined with a conventional amino acid analyzer. These modifications increase the sensitivity to that of microbore analyzers equipped with ninhydrin detection systems[4,9] and allow the radioactivity to be measured in the spectrofluorometer effluent directly.

ACKNOWLEDGEMENT

This research was supported in part by NIH Grant AM1911601.

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