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Spectrophotometric Assay for Ornithine Decarboxylase[1]

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A rapid and sensitive spectrophotometric assay for ornithine decarboxylase is described. It is based on the observation that the product of ornithine decarboxylase, putrescine, reacts with 2,4,6-trinitrobenzenesulfonic acid to give a colored product soluble in 1-pentanol whereas ornithine does not. The amount of putrescine produced by the enzyme was determined by measuring the absorbance of the 1-pentanol extract of the reaction mixture at 420 nm, and by comparing the results to those obtained by the trapping of $^{14}CO_2$ and by HPLC assays. The three assays were found to be equivalent in sensitivity, with the spectrophotometric assay having the advantages of being relatively rapid, requiring only common laboratory equipment, and not requiring the use of radioactive isotopes.

KEY WORDS: ornithine decarboxylase; decarboxylases; polyamines; trinitrobenzene sulfonic acid; spectrophotometry; cancer diagnosis.

Ornithine decarboxylase (ODC[3]; EC 4.1.1.17) and *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) are key enzymes in the biosynthesis of the ubiquitous polyamines putrescine (1,4-diaminobutane), spermidine [$NH_2(CH_2)_3NH(CH_2)_4NH_2$], and spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$]. Because the levels of these enzymes, as well as those of the polyamines, are observed to increase upon the initiation of cell proliferation (1,2,3), the assay of their activities has become an important analytical tool. For example, increased levels of polyamine and ODC are one of the first events detectable upon transformation of cells to neoplasia (4); hence an increase in ODC activity provides one of the most reliable markers for the clinician in evaluating the progression of tumors (5).

In this paper we describe a simple, sensitive, inexpensive, nonradioactive, and accurate spectrophotometric assay for ODC. This assay can also be used to measure putrescine. The assay is based upon previous spectrophotometric assays developed in our laboratory for lysine decarboxylase (6) and tyrosine decarboxylase (7). The assays are based on the observation that picrylsulfonic acid (2,4,6-trinitrobenzenesulfonic acid, TNBS) reacts with amines and the amino groups of amino acids at alkaline pH (8) to give colored trinitrophenyl (TNP) adducts having molar extinction coefficients greater than 10^4 (9).

Our spectrophotometric assay for ornithine decarboxylase (Scheme 1) is based on the observation that the TNP adduct of ornithine is soluble in water, but not in 1-pen-

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[3] Abbreviations used: ODC, ornithine decarboxylase; SAMDC, *S*-adenosylmethionine decarboxylase; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNP, trinitrophenyl; DMSO, dimethyl sulfoxide; PPO, 2,5-diphenyloxazole.

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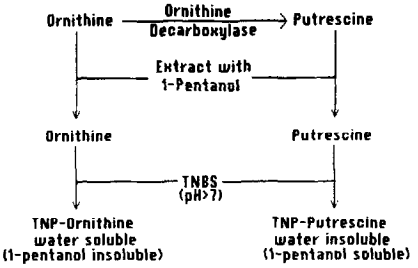

SCHEME 1

tanol, whereas for TNP-putrescine the reverse is true.

MATERIALS AND METHODS

Reagents and Instruments

For preparing sample. Ornithine decarboxylase [EC 4.1.1.17, from *Escherichia coli* and *Neurospora crassa* (10)], β-mercaptoethanol, ethylenediaminetetracetic acid (EDTA), pyridoxal-5-phosphate, L-ornithine HCl (Sigma Co.); perchloric acid (Mallinckrodt); and DL-[1-^{14}C]ornithine (40K cpm per microliter, ICN).

For spectrophotometric assay. 1-pentanol, dimethyl sulfoxide (DMSO, J. T. Baker Co.); putrescine dihydrochloride, L-ornithine HCl, and picrylsulfonic acid (trinitrobenzenesulfonic acid, Sigma Co.). All optical measurements were made using a Beckman Acta C-III spectrophotometer.

For trapping $^{14}CO_2$. Triton X-100, toluene (Mallinckrodt); 2,5-diphenyloxazole (PPO, National Diagnostic); monoethanolamine (95%), methylcellosolve (Aldrich); and No. 1 filter paper (Whatman). Scintillation was measured using a Beckman LS-230 liquid scintillation counter.

For HPLC measurements. Dansyl chloride, putrescine dihydrochloride (Sigma Co.); acetone (HPLC grade), ethyl acetate (Mallinckrodt); acetonitrile (HPLC grade, VWR); and 0.45-μm membrane filters (Alpha-450, Gelman). Separation was performed on a Gilson high-performance liquid chromatography system using an Altex Ultrasil ODS column (0.46 × 4.5 cm, particle size 10 μm). Effluent was monitored using a Gilson Spectra/Glo filter fluorometer; data were integrated with a Gilson Datamaster (11).

Methods

For preparing samples. Ornithine decarboxylase from *E. coli* was purified by separating the commercial enzyme through No. 3ES and No. 3FS Sephadex columns, giving a final protein concentration of 2.0 mg/ml. The *N. crassa* enzyme was a crude extract of a culture highly derepressed for the enzyme (10). Before use the enzyme solution was diluted to give final volumes of 100 μl. For the spectrophotometric and HPLC assays, the substrate solutions consisted of 2.5 mM β-mercaptoethanol, 1.5 mM EDTA, 75 nM pyridoxal-5-phosphate, and 3 mM L-ornithine HCl in 150 mM potassium phosphate (pH 7.1). A similar substrate solution containing 1% (v/v) DL[1-^{14}C]ornithine (11) was prepared for the assay involving the trapping of radioactive CO_2. The reaction was initiated by the addition of 200 microliters of substrate solution to the 100-μl sample of diluted enzyme sample; it was terminated after 30 min at 37°C by adding 200 μl of 1 M perchloric acid or 200 μl of 10% trichloroacetic acid. The reaction mixture was then assayed for its content of putrescine.

Spectrophotometric assay. Ornithine and putrescine standards were prepared in 0.1 M phosphate buffer (pH 7.0). One ml of 4 N NaOH was added and mixed vigorously to 0.5-ml aliquots of standards or the terminated reaction mixtures. Two milliliters of 1-pentanol was then added and the samples were mixed vigorously for 20 s with a Vortex mixer. After the emulsions were centrifuged for 5 min at 2000 rpm, 1 ml of the upper (organic) phase was transferred to test tubes containing 1 ml of 0.1 M sodium borate (pH 8.0) and were mixed briefly. One milliliter of 10 mM TNBS dissolved in 1-pentanol was added to each tube, and the samples were

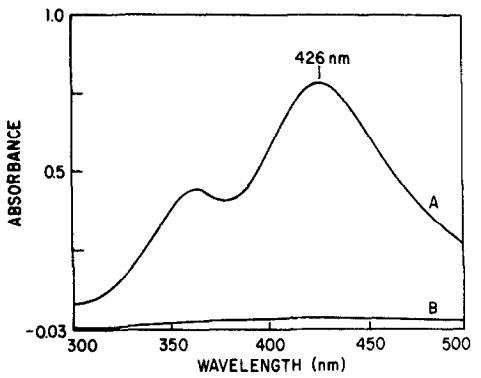

FIG. 1. Absorption spectra of TNP-putrescine (A) and TNP-ornithine (B) extracted in 1-pentanol.

mixed for 20 s with a Vortex mixer; 2 ml of DMSO were then added to each tube and the samples were mixed for an additional 20 s. The phases were separated by centrifuging the sample for 5 min at 2000 rpm, and the absorbance of the organic phase of each sample was measured against a reagent blank.

Trapping of $^{14}CO_2$. Prior to the addition of [^{14}C]ornithine, filter paper fans were spotted with 10 μl of monoethanolamine:methylcellosolve (1:2 v/v) and placed in the mouths of 13 × 100-mm tubes containing dilutions of the enzyme. The tubes were stoppered during the assay and for 90 min at 37°C after the addition of trichloroacetic acid. The fans were then placed in 6-ml betavials with 0.7 ml of water and 4 ml 1:2 Triton:PPO (5 mg/ml in toluene) and counted using the scintillation counter.

HPLC measurements. One hundred microliters of putrescine standards and enzyme reaction samples were incubated overnight at room temperature with 100 μl of dansyl chloride (5 mg/ml in acetone) in the presence of saturating sodium carbonate. Samples were then extracted with 0.2 ml of ethyl acetate and filtered through 0.45-μm membrane filters before applying them to the HPLC column. The elution program began with 50% acetonitrile in H_2O, increased linearly for 7.5 min to 95% acetonitrile, and then decreased to 50% over 3 min. An internal standard, 1,7-diaminoheptane, was used throughout.

RESULTS

Absorption Spectra

The absorption spectra of 1-pentanol extracts of the products of the reaction of TNBS with 0.5 mM ornithine and 0.5 mM putrescine are shown in Fig. 1. TNP-putrescine has a small absorption peak with a maximum at 360 nm and a major peak with maximum absorbance at 426 nm. The 1-pentanol extract of TNP-ornithine shows no significant absorbance within this range.

Standard Curve and Extinction Coefficient for Putrescine

Varying concentrations of TNP-putrescine extracted in 1-pentanol gave the standard curve shown in Fig. 2. The curve was linear ($r > 0.99$) in the range of 25 to 500 micromolar. The average molar extinction coefficient was found to be $1.91 \pm 0.58 \times 10^3$ M^{-1} cm^{-1}.

Assay of Ornithine Decarboxylase

Ornithine decarboxylase activity was measured for enzyme concentrations ranging

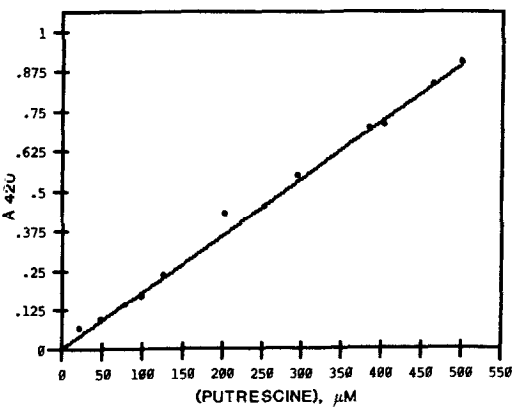

FIG. 2. Standard curve for determining varying concentrations of putrescine. Readings were obtained from 1-pentanol extracts of increasing concentrations of TNP-putrescine (25 to 500 μm) prepared as described under Materials and Methods. Pure 1-pentanol was used as a blank.

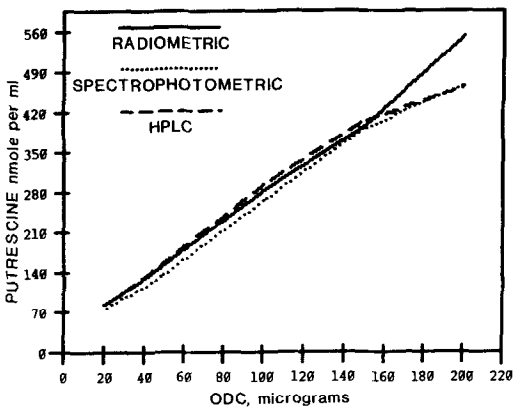

FIG. 3. Assay of ornithine decarboxylase activity by radiometric (—), HPLC (---), and spectrophotometric (· · ·) assays.

from 20 to 200 μg/ml. The amount of putrescine formed in 30 min was assayed by the spectrophotometric, $^{14}CO_2$ trapping, and HPLC methods (Fig. 3). Replots of these slopes against one another show that the highest degree of correlation exists between the HPLC and spectrophotometric assays (slope = 0.97, $r > 0.997$), followed by the $^{14}CO_2$ trapping and spectrophotometric (slope = 1.13, $r > 0.989$), and $^{14}CO_2$ trapping and HPLC assays (slope = 1.15, $r > 0.983$).

DISCUSSION

All three assays for ornithine decarboxylase activity appear to be equivalent in terms of sensitivity and linearity over a range of enzyme concentrations, particularly between 20 and 150 μg. The correlation between the results of the HPLC and spectrophotometric assays are particularly striking. These three assays, however, are not equivalent in terms of equipment requirements and procedural complexity. The $^{14}CO_2$ trapping method involves the use of radioactive isotopes and requires care to avoid contamination of the base-treated-fan with substrate or acid. The HPLC method requires expensive equipment that is not available in most laboratories. On the other hand, the spectrophotometric assay requires no special equipment, does not use radioisotopes, is extremely rapid, and is equivalent in sensitivity to both the HPLC and the $^{14}CO_2$ trapping methods.

ACKNOWLEDGMENTS

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