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Note

Simultaneous assay of choline kinase and choline oxidase in tissue by high-performance cation-exchange chromatography and continuous radioactive detection*

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The initial step in the synthesis of phosphatidylcholine (PC) from choline via the CDP–choline pathway depends on the phosphorylation of choline by choline kinase (ATP: choline phosphotransferase, EC 2.7.1.32) to form phosphorylcholine[1]. This enzyme, present in cytoplasmic fractions, is particularly active in developing tissue or in tissues exhibiting rapid growth[2]. Its importance lies in its rate limiting control of PC synthesis[3] and sensitivity to insulin stimulation in some tissues[2,4]. Choline is also oxidized to betaine by the mitochondrial enzyme choline oxidase (choline: oxygen 1-oxido-reductase, EC 1.1.3.17)[5] and in neural tissue acetylated to acetylcholine by choline acetyltransferase (Acetyl-CoA: choline O-acetyltransferase, EC 2.3.1.6)[6]. The activity of each of these enzymes for the common substrate choline varies significantly, depending on the type of preparation, age of the tissue, and tissue source[2,3,5]. The assay of choline kinase activity may underestimate the true activity in tissues or preparations with significant contributions of choline oxidase or choline acetyltransferase activity making it necessary to measure all possible reaction products.

Typically the analysis of these enzymatic products has been difficult, requiring the extraction of phosphorylcholine into a Reineckate solution and separation of reaction products by thin-layer or paper chromatography[7,8]. The separation of choline metabolites by high-performance liquid chromatography (HPLC) is hindered by a lack of UV absorption and therefore a lack of adequate detection.

In the current study we have developed an HPLC separation technique based on an isocratic cation-exchange separation of [^{14}C]choline and its phosphorylated, oxidized and acetylated enzymatic products utilizing a radioactive flow detector which allows for a rapid and sensitive quantitation in the nanomolar range with reproducibility and accuracy far exceeding available paper chromatography techniques.

* The views of the authors do not purport to reflect the views of the Department of the Army or the Department of Defense.

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EXPERIMENTAL*

Apparatus

This study was performed using a Spectra Physics (San Jose, CA, U.S.A.) Model 8100 programmable liquid chromatograph, equipped with a Model 8110 autosampler, a 10-μl loop injector and a Model 4270 two-channel recording integrator. Radioactive detection was accomplished on line with a Radiomatic Instruments (Tampa, FL, U.S.A.) Model FLO-ONE programmable radioactive flow detector.

Reagents

Sodium hydroxide, citric acid, choline kinase, choline oxidase and choline chloride were obtained from Sigma (St. Louis, MO, U.S.A). [^{14}C-methyl]choline chloride and [^{14}C-methyl]acetylcholine iodide were supplied by New England Nuclear (Boston, MA, U.S.A.). Radiomatic Instruments supplied the scintillation fluid, Flo-Scint III.

Procedure

Column preparation. An empty column, 15 cm × 3.9 mm I.D. was packed by the upflow method with Chromobeads Type B, T15-0355-42, (Technicon, Terrytown, NY, U.S.A.) in 0.2 *N* sodium hydroxide at pressures up to 5000 p.s.i. This packing is a cation-exchange medium that is suitable for use over a wide pH range (2–10) and of withstanding high operating pressures and temperatures.

Separation and detection. The mobile phase, 0.6 *M* sodium citrate, was prepared fresh daily by titrating sodium hydroxide, 22.750 g/l, with citric acid to a pH of 6.3. The mobile phase concentration was not critical and a variation of 0.05 molar units did not affect retention times. Flow-rates were maintained at 1.0 ml/min at a column temperature of 54°C. Pressures ranged between 1400 and 1800 p.s.i. Samples were introduced through a 10-μl continuous flow loop injector. The column effluent passed directly into the FLO-1 radioactivity detector containing a 0.5-ml flow cell. Effluent and scintillant were mixed 1:3 with a resultant efficiency of 84% for ^{14}C and detection sensitivity of 50 dpm. Output was automatically corrected for efficiency and flow-rate and reported as the total integrated dpm/peak. In this configuration less than 10 ng of labelled compounds could be detected.

Enzyme assay and sample preparation. Radioactive standards of betaine and phosphorylcholine were prepared by a modification of the method described by Ulane *et al.*[7] by incubating [^{14}C-methyl]choline with yeast choline kinase (Sigma) and choline oxidase (Sigma) respectively in a reaction buffer containing 67 m*M* Tris–HCl (pH 8.5), 10 m*M* dithiothreitol, 12 m*M* MgATP, 11 m*M* magnesium chloride, 5 m*M* choline chloride and 0.2 μCi/ml of [^{14}C-methyl]choline chloride (specific activity 55 mCi/mmole). Total volume of the reaction was 600 μl. The reaction was carried out for 1 h at 37°C and stopped by the addition of 150 μl of glacial acetic acid. Reaction products were separated by HPLC and the peaks collected manually.

Enzyme asssays were performed on crude homogenates of cultured toad kidney epithelia (line A6) utilized for their sensitivity to insulin and aldosterone[9]. The cells

* The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

were scraped from Petri dishes into homogenization buffer (50 mM Tris pH 8.0, 1 mM EDTA, 5 mM dithiothreitol) and homogenized in a 1-ml Wheaton–Dounce glass homogenizer. The homogenate was then centrifuged at 3000 g for 10 min and the supernatant was assayed for enzyme activity. Protein concentration was determined by the method of Bradford[10].

RESULTS AND DISCUSSION

The usual methods for choline kinase measurements involve the precipitation of choline from phosphorylcholine by saturated Reineckate solution followed by paper chromatography of reaction products or the extraction directly of reaction products and separation by paper chromatography[2,7,8]. Since most tissue extracts contain both choline kinase and choline oxidase activity, with k_M values for choline which could result in competition for the substrate[2] (Fig. 1), it is desirable to measure activities of both enzymes unless partial purification of the enzyme is undertaken. In an effort to find a reasonably rapid and accurate assay for choline kinase in tissue we have separated reaction products by cation-exchange HPLC coupled with online radioactive detection.

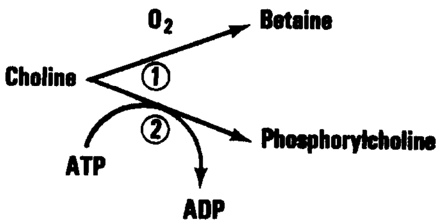

Fig. 1. Pathway for the reaction of choline by (1) choline: oxygen 1-oxido-reductase (EC 1.1.3.17), and (2) ATP: choline phosphotransferase (EC 2.7.1.32).

Fig. 2 is a representative chromatogram showing clean separations of [^{14}C-methyl]choline from radioactive standards phosphorylcholine, betaine, and acetylcholine. The complete separation of choline from phosphorylcholine can be accomplished in approximately 22 min. In other experiments not shown here, a column of 10 cm length operated at the same temperature at 1.5 ml/min yielded complete, isocratic separations in less than 15 min. The reproducibility of this method is extremely high with less than 2% variation on repeat injections. Since in most tissue assays for choline kinase less than 3% of the substrate is converted to phosphorylcholine, extremely accurate and sensitive detection is necessary for small quantities of tissue extracts. Fig. 3 compares the time courses of an incubation reaction with the toad kidney epithelia homogenates. Unlike the method described by Ulane *et al.*[7] it was not necessary to concentrate the extract. Separate betaine and phosphorylcholine peaks increase with reaction time. This reaction was linear with protein and incubation time, as long as the reaction did not consume more than 75% of the [^{14}C-methyl]choline substrate. The activity of choline kinase in these cells is 4.52 ± 0.90 nM/min/mg protein. In an effort to determine if both enzymes could compete for the same substrate, partially purified commercial choline kinase and choline oxidase were

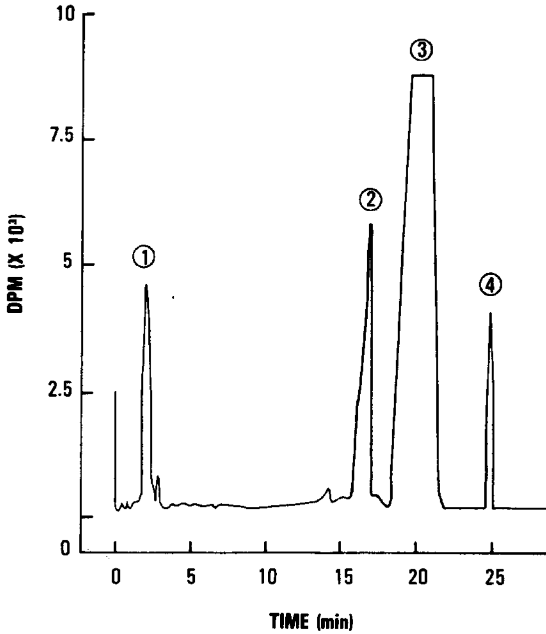

Fig. 2. Representative radioactive separation of a standard solution containing (1) phosphorylcholine, (2) betaine, (3) choline and (4) acetylcholine.

incubated alone and together with equivalent amounts of substrate. The time course of the reaction comparing each activity alone and in combination is shown in Fig. 4. When the activity for choline kinase alone (116 ± 4 μM/min) is compared to its activity in combination with choline oxidase (110 μM/min) there is no difference. The

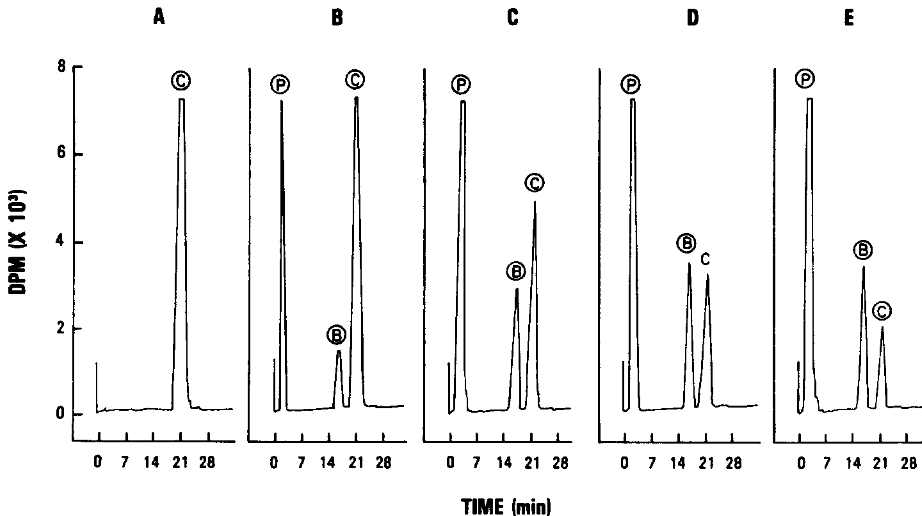

Fig. 3. Radioactive chromatograms of incubated tissue homogenates at incubation times of (A) 0 min, (B) 15 min, (C) 30 min, (D) 60 min, and (E) 90 min. Peaks are phosphorylcholine (P), betaine (B) and choline (C).

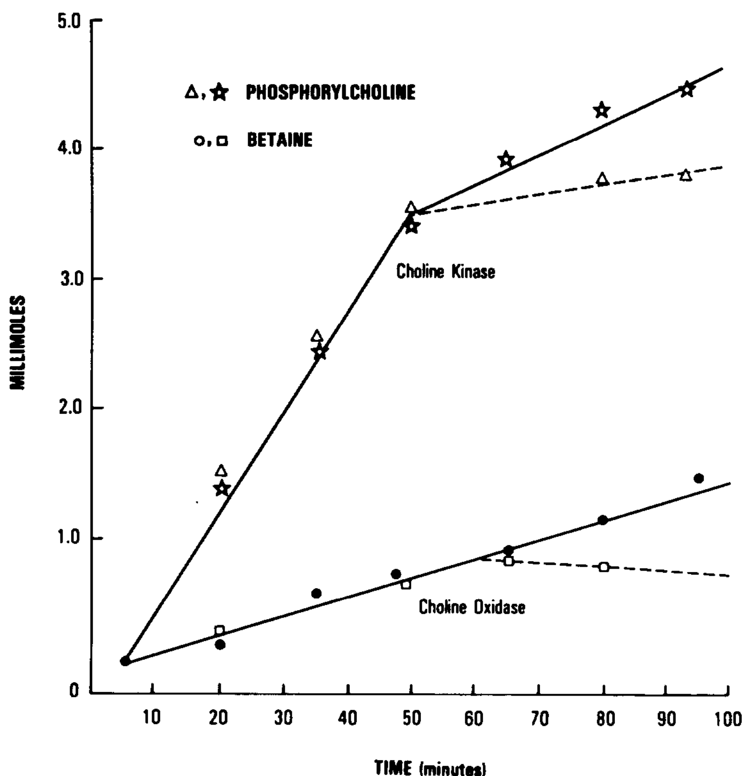

Fig. 4. Phosphorylcholine and betaine formed with increasing reaction time at a constant substrate concentration of 5 μM. (☆) and (●) are the enzymes alone while (△) and (□) are the enzymes incubated together at the same activities.

reaction *versus* time, shown in Fig. 4, for choline kinase alone or in combination with choline oxidase is essentially identical until approximately 75% of the substrate is consumed. Likewise, choline oxidase activity (33.3 alone *versus* 31.3 μM/min in combination with choline kinase) is unaffected by the presence of choline kinase as long as sufficient substrate is present in the reaction buffer. The reactions were also linear with protein concentration, as long as sufficient substrate was present.

This method provides a relatively rapid assay of both choline kinase and choline oxidase in relatively crude tissue homogenates with sufficient sensitivity to assay the homogenates directly without extensive sample preparation, dialysis or enrichment. The sensitivity of the method permits enzyme analysis of small quantities of cultured cells and is well suited to study potential regulators of choline kinase activity.

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