nous unlabeled folate distribution is of interest. Although this is useful as a measure of any losses that may occur during the various manipulations, as indicated above, the recovery obtained is not necessarily an accurate indicator of the effectiveness of every step in the procedure.

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[51] Detection of *p*-Aminobenzoylpoly(γ-glutamates) Using Fluorescamine

By PETER C. LOEWEN

The analysis of natural folate pools has been complicated by both the multiplicity of oxidation states and the multiplicity of polyglutamate lengths. Methods have been developed for the cleavage of the folate mixture to create a *p*-aminobenzoylpoly(γ-glutamate), pABAglu$_n$, fraction in which the polyglutamate chain lengths have been characterized by ion-exchange[1-5] and polyacrylamide gel[6] chromatography. In order to increase the sensitivity of the assay of these column eluates without resorting to radioactive precursors, fluorescamine, which forms fluorescent adducts with amines, can be used.[7] The fluorescamine adduct of *p*-aminobenzoic acid (pABA) and its polyglutamate derivatives exhibit 50- to 100-fold greater fluorescence than similar adducts of other amines such as amino acids, thus allowing the detection of picomole amounts of pABA derivatives.[7]

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Preparation and Chromatography of the pABAglu$_n$ Fraction

Principle. The folate pool is extracted and the pABAglu$_n$ portion is removed by reductive cleavage. The pABAglu$_n$ mixture can be directly fractionated on DEAE-Sephadex A-25 or partially purified by reversible conversion to an azo dye[6,8,9] prior to ion-exchange separation.

Procedure. Bacteria and fungi were collected by centrifugation and washed once in 0.1 *M* potassium phosphate (pH 7.6). Pellets containing 1–5 g of cells were weighed, resuspended in 10 ml of the same buffer, and boiled for 5 min. Tissue extracts were prepared by homogenization in 0.2 *M* HCl. The debris was removed by centrifugation, and the supernatant brought to pH 1.0 using concentrated HCl and stored overnight at 4°. After readjusting the pH to 6.0 with 2 *M* NaOH, 0.5 g of $NaBH_4$ was added and the mixture was shaken for 15 min. Excess $NaBH_4$ was destroyed by acidification and 1 g of powdered zinc was added, followed by shaking at 20° for 5 min. After the zinc was removed by centrifugation the solution was neutralized and immediately charged onto a 0.7 × 50-cm DEAE-Sephadex A-25 column equilibrated with 0.15 *M* NaCl in 0.04 *M* potassium phosphate (pH 7.6). In order to ensure reproducibility and to eliminate the possibility of contaminating peaks, all resin was pretreated first with 0.5 *M* HCl and then with 0.5 *M* KOH. The resin was washed with distilled water after each treatment and then equilibrated with buffer. A new column was prepared for each determination. After the sample was charged, the column was washed with 50 ml of equilibrating buffer and a linear gradient was applied by mixing 250 ml of 0.15 *M* NaCl–0.04 *M* potassium phosphate (pH 7.6) with 250 ml of 0.7 *M* NaCl in the same buffer. Fractions of 2.5 ml were collected for reaction with fluorescamine.

Alternatively, if partial purification of the pABAglu$_n$ fraction was required, the solution from zinc cleavage could be treated to form diazo derivatives of the pABAglu$_n$ component by the sequential addition at 2-min intervals of 4 ml of 0.5% sodium nitrite, 4 ml of 2.5% ammonium sulfamate, and 2 ml of 0.1% naphthylethylenediamine. After 20 min at 20°, a 4-ml suspension of Dowex AG-50 (200–400 mesh) in 0.2 *M* HCl was added and shaken for 5 min. The suspension was loaded into a 1.3 × 3.0-cm column and washed with 40 ml of 0.2 *M* HCl followed by 30 ml of 0.1 *M* potassium phosphate (pH 7.6) and 30 ml of H_2O. The purple diazo dye was reduced and the pABAglu$_n$ was eluted from the column with 10 ml of 0.3 *M* sodium hyposulfite. Fractions of 1 ml were collected from which 10

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μl were taken and added to 1 ml of 0.1 *M* potassium phosphate (pH 7.6) for reaction with fluorescamine. The material that reacted with fluorescamine eluted as a single peak and these fractions were pooled and concentrated to dryness. After resuspension in 10 ml of 0.15 *M* NaCl in 0.04 *M* potassium phosphate (pH 7.6), the solution was charged onto a column of DEAE-Sephadex A-25 as described above.

Comments. The amount of cell paste or tissue required for a satisfactory elution profile depended on the organism, but routinely 1 g or more was a good starting point for a first analysis of any organism. The direct application of the zinc-reduced material to the ion-exchange resin avoided a 35–40% loss of material inherent in the recovery from the diazo adduct. In addition, there was usually no enhancement of background fluorescence in the elution profile, with the only difference in results between the two procedures being the greater amount of material in the nonderivatized eluates.

Detection of $pABAglu_n$ Using Fluorescamine

Principle. Fluorescamine reacts rapidly with the amino group of *p*-aminobenzoic acid and its polyglutamate derivatives to form a highly fluorescent adduct. Portions of the eluate are treated with fluorescamine to determine the location and amount of the various sizes of $pABAglu_n$ present.

Procedure. For each set of assays, a fresh solution of 3 mg/ml fluorescamine was prepared in acetone dried over sodium sulfate. To 1 ml of sample volume, 15 μl of the fluorescamine solution was added followed by gentle mixing. After 5 min at 20° the relative fluorescence of the solution was determined using an excitation wavelength of 400 nm and an emission wavelength of 500 nm. A standard value of fluorescent units per nanomole of pABA can be determined by reacting increasing amounts of pABA with fluorescamine in the same assay system. This standard value can then be used to quantitate the amount of $pABAglu_n$ in each peak in the elution profile.

Comments. Because of the intense fluorescence of the fluorescamine–pABA adduct relative to most other fluorescamine adducts, other naturally occurring amines did not interfere with the assay. The relative fluorescence of the fluorescamine–pABA adduct was unaffected by changes in the pH between 5.8 and 9.2, whereas the fluorescence of the amino acid adducts dropped as the pH was lowered.[7] In determining the standard value, which is best done for each series of quantitative assays, as little as 25 pmol/ml of pABA could be detected in a direct assay. However, dilution of the sample during chromatography made it necessary to have at least 500 pmol of a species present for accurate quantitation following elution from the ion-exchange resin. The length of the polyglutamate chain did not affect the relative fluorescence of the pABA–fluorescamine adduct.

[52] Preparation and Analysis of Pteroylpolyglutamate Substrates and Inhibitors

By ROWENA G. MATTHEWS

In recent years, there has been considerable interest in the role of folyl- and antifolylpolyglutamates in cellular metabolism.[1,2] A number of research groups are measuring K_i values associated with inhibition of folate-dependent enzymes by pteroylpolyglutamates or kinetic parameters associated with catalysis involving pteroylpolyglutamate substrates. Since the pteroylpolyglutamates typically bind more tightly to their target enzymes than do the corresponding monoglutamates, it is essential that preparations of pteroylpolyglutamate substrates and inhibitors be free of degradation products, and that they be homogeneous preparations of defined polyglutamate chain length and of defined structure at the pteridine. Tetrahydropteroylpolyglutamate derivatives should be prepared by enzymatic reduction to avoid contamination with the unnatural C-6 diastereomer, which may be a potent inhibitor. In addition, since the pteroylpolyglutamates which serve as starting materials for these preparations are expensive and available only in limited quantities, it is highly desirable that the preparation of pteroylpolyglutamate substrates and inhibitors proceed with high yields.

In this article, I shall describe methods of preparation and analysis which we have developed for the production of $PteGlu_n$, $H_2PteGlu_n$, $H_4PteGlu_n$, $5,10\text{-}CH_2\text{-}H_4PteGlu_n$, and $5\text{-}CH_3\text{-}H_4PteGlu_n$ derivatives of the requisite purity, using as starting materials the appropriate $PteGlu_n$ derivatives prepared by solid-phase synthesis.[3] Pteroylpolyglutamates ($PteGlu_n$)

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