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IMMOBILIZED ENZYMES IN THE PRODUCTION OF RADIOPHARMACEUTICALLY PURE AMINO ACIDS LABELED WITH ^{13}N

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Nitrogen-13 and ^{11}C compounds may be obtained by enzymatic synthesis but the frequent presence of potentially pyrogenic and antigenic enzymes in the final product creates problems of radiopharmaceutical purity. This report describes methods for the synthesis of pharmaceutical-quality ^{13}N-labeled amino acids by binding the required enzymes to a solid-state support. Nitrogen-13-L-glutamic acid was synthesized on stream by using glutamic acid dehydrogenase which had been immobilized on porous derivatized (N-hydroxysuccinimide) silica beads and packed into a column. Nitrogen-13-L-alanine was synthesized by adding pyruvate to ^{13}N-L-glutamic acid and passing this mixture through a second column of similar silica beads containing the immobilized enzyme glutamic–pyruvic transaminase.

The advent of the biomedical cyclotron has resulted in a renewed interest in the ultra-short-lived isotopes ^{11}C ($T_{1/2}$ = 20 min) and ^{13}N ($T_{1/2}$ = 10 min). These nuclides are required for the labeling of many small biologically active compounds where an externally detectable label is required. Many compounds have recently been labeled with ^{11}C or ^{13}N (*1–10*). These compounds have been synthesized by either ordinary "wet chemistry" techniques (*1–3*) or by enzymatic synthesis (*4–10*). Nitrogen-13-L-alanine has previously been synthesized by a nonspecific enzymatic reaction (*10*).

"Wet chemistry" techniques are relatively slow, produce intermediate compounds, and yield racemic mixtures of compounds having optical isomers. An enzymatic synthesis is rapid, produces no intermediate compounds, and yields the specific end product. The use of enzymes, however, creates problems in obtaining products of radiopharmaceutical purity. This report concerns the synthesis of pharmaceutical-quality ^{13}N-L-glutamic acid and ^{13}N-L-alanine by binding the two required enzymes on solid-state supports so that the end product is free of the potentially antigenic and pyrogenic enzymes. Terminal sterilization is then accomplished by Millipore filtration.

MATERIALS

The enzymes, glutamic acid dehydrogenase (EC 1.4.1.2), and glutamic acid–pyruvic acid transaminase (EC 2.6.1.2), were purchased from Boehringer Mannheim Corp. Sodium pyruvate was obtained from Nutritional Biochemicals Corp., reduced nicotinamide diphosphopyridine nucleotide (NADH), Tricine and alpha-ketoglutaric acid from Calbiochem, and the ion-exchange resins, AG 50W-X8 and AG 1-X8, from BioRad Laboratories. Porous silica beads were obtained from Pierce Chemical Co. (CPG/N-Hydroxysuccinimide). Other chemicals used were reagent grade.

METHOD

Reagents and glassware are prepared in advance and checked for sterility and apyrogenicity prior to use. Reagents are packaged in single-dose vials.

The enzymes are immobilized by covalent binding to derivatized silica beads. This is accomplished by adding the enzyme (800 units) in 10 ml of 0.1 *M* Tricine buffer, pH 8.2, and 1×10^{-5} *M* EDTA to 0.8 gm of beads. The mixture then is degassed. The mixture is refrigerated (4°C) and agitated every 30 min for 2 hr and then refrigerated overnight without agitation. The following morning the beads are

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washed six times with a total of 100 ml of 0.5 *M* glycine and the beads are then packed into a column (0.7 × 11 cm). The column is wetted with 0.05 *M* Tricine buffer, pH 8.2, containing 5×10^{-3} *M* EDTA and kept under refrigeration until ready for use.

Nitrogen-13-NH_3 is obtained from the UCLA Biomedical Cyclotron by the irradiation of water using a (p,α) reaction (*11*). Nitrogen-13-ammonia is trapped in 0.5 ml of a buffer solution containing 0.1 *M* Tricine buffer, pH 8.2, and 1×10^{-5} *M* EDTA. The ^{13}N-ammonia solution is made to a final volume of 1 ml containing 10 μM of alpha-ketoglutarate, 1 μM of NADH, 100 μM of Tricine buffer, pH 8.2, and 0.01 μM of EDTA. This mixture is then passed through a reaction column (0.7 × 11 cm) containing the immobilized enzyme, glutamic acid dehydrogenase. Pressure is applied to this and subsequent columns for rapid elution by using a peristaltic pump to maintain a flow rate of 4 ml/min. One hundred microliters of 0.1 *M* pyruvic acid is then added to the 2 ml of eluate containing newly formed ^{13}N-L-glutamic acid and this mixture is passed through a second reaction column containing the immobilized enzyme, glutamic–pyruvic transaminase which transaminates the ^{13}N-L-glutamic acid to form ^{13}N-L-alanine.

The eluate of the second reaction column (2.5 ml) is added to an AG 50W-X8 column (0.7 × 11 cm) equilibrated at pH 4.9. The $^{13}NH_4+$ and other positively charged ions are bound to the column. The ^{13}N-labeled amino acids are eluted with 0.01 *M* Tris buffer, pH 4.9. A flow monitor is used to determine the location of the radioactive peak. Seventy to 75% of the radioactivity which is applied to the column is thus eluted in 5 ml. The bulk (60–65% of the total radioactivity) of the eluted ^{13}N-amino acids is present in 3–4 ml. The latter solution contains both ^{13}N-L-glutamic acid and ^{13}N-L-alanine. These amino acids are separated on an AG 1-X8 ion-exchange column (0.7 × 11 cm) equilibrated at pH 7.5. The eluate containing the ^{13}N-amino acids is added to this column. Sodium acetate buffer (0.05 *M*), pH 7.5, is then used for elution. Nitrogen-13-L-glutamic acid is bound to the column while the ^{13}N-L-alanine passes through. This radioactivity, 60–70% of that added to the column, is present in 6–8 ml of solution. The ^{13}N-L-alanine is then sterilized by passage through a 0.22-micron Millipore filter.

The columns containing the immobilized enzymes are then washed with 50 ml of buffer solution containing 0.05 *M* Tricine buffer, pH 8.2, and 5×10^{-3} *M* EDTA. The columns wetted with this buffer are aseptically stoppered and stored at 4°C for later reuse. About 1 hr prior to reuse, the buffer solution is eluted and tested for endotoxin using the Limulus test. The resin columns, which aid in both separation and identification of the reaction products, are not reused but may be recycled.

RESULTS AND DISCUSSION

We have previously prepared ^{13}N-labeled amino acids by incubation of the substrate, soluble enzyme, and cofactors in a test tube with subsequent separation by column chromatography (*9*). If the column is permitted to elute by gravity, the enzyme mobility is retarded and the enzyme appears to be bound to the top of the column. The unique time demands of working with a 10-min half-life isotope require more rapid elution. Pressure is, therefore, applied to the column. While this gives more rapid elution through the column, it also causes the enzyme to pass through the column and appear in the final product. These enzymes are potentially pyrogenic and antigenic. Nitrogen-13-labeled amino acids containing enzyme have been successfully utilized in animals for tissue-distribution and imaging studies (*5,6,8–10*) but human studies obviously require a pharmaceutical-quality preparation.

The term "immobilized enzyme" refers to enzymes that are synthetically attached to a water-insoluble support by either physical or chemical means. The first such synthetically immobilized enzymes were reported in 1953 by Grubhofer and Schleith (*12,13*) using a polyaminostyrene. The covalent attachment of an enzyme to an insoluble derivatized glass support was first reported in 1969 by Weetall (*14,15*). Although the support is insoluble, the enzyme may retain its solubility though its movement is restricted to a microspace. Immobilized enzymes provide a large available surface area for the reaction to occur and there is less steric interference than with test tube incubation. Detailed discussion of immobilized enzymes on various supports may be found in the monograph by Zaborsky (*16*).

Immobilization of the enzymes on a solid-state support has permitted us to synthesize ^{13}N-L-glutamic acid and ^{13}N-L-alanine free of enzyme as measured by a micromodification of the Lowry technique (*17*). The use of immobilized enzymes has also resulted in other improvements. The two-step alanine synthesis is performed on stream and requires only about 4 min whereas incubation in a test tube required 10 min. Higher yields were obtained using the immobilized enzymes (70–75%), as compared with test tube incubation (50–60%). Covalent attachment of an enzyme to an insoluble support produces a derivatized enzyme which may have different chemical or physical properties or both from the native enzyme, e.g., catalytic activity may be en-

hanced or decreased. The increased yield of L-alanine despite the shortened reaction time may be due to the larger amount of enzyme used on the solid-state support (800 units versus 20 units in test tube incubation) but other factors cannot be excluded.

The ^{13}N-L-glutamic acid obtained from the first enzymatic reaction may be utilized as glutamic acid or may be transaminated to form ^{13}N-L-alanine by the second enzymatic reaction. Immobilization of the two enzymes on separate supports permits us to retain this flexibility. The covalent attachment of multiple other enzymes to a single water-insoluble support has been reported, however (*16*).

Enzymes immobilized on the beads are reusable. When not in use the enzyme beads are wetted with 0.5 *M* Tricine buffer, pH 8.2, containing 5×10^{-3} *M* EDTA and stored under refrigeration. Prior to reuse, the wetting solution is checked for endotoxin using the Limulus test. The beads are washed with 50 ml of 0.1 *M* Tricine buffer, pH 8.2, containing 1×10^{-5} *M* EDTA before initiating the reactions.

Glutamic acid dehydrogenase has been immobilized on different types of solid-state supports (*18,19*), but silica beads appear to be particularly suitable for this enzyme and other enzymes to be used in studies with short-lived nuclides. Silica beads may be derivatized readily and used under pressure without being compressed. Since reactions involving short-lived radioisotopes must be completed in a short time period, pressure must be applied to increase flow through the beads over the flow rate possible with gravity alone. Derivatized Sephadex supports have been found by us to collapse under pressure.

Reaction conditions are established using ^{14}C substrates before attempting a ^{13}N synthesis. This not only permits selection of optimal pH, volumes, and concentration of ingredients but also permits selection and calibration of the chromatographic ion-exchange columns. The AG 50W-X8 columns at pH 4.9 will adsorb all positively charged ions such as $^{13}NH_4+$. The AG 1-X8 column at pH 7.5 adsorbs the negatively charged ^{13}N-L-glutamic acid, alpha-ketoglutarate, and pyruvic acid but permits ^{13}N-L-alanine to pass through. The 10-min half-life of ^{13}N does not permit time for chemical analysis of the product of each reaction. The ion-exchange columns and the nature of the starting materials limit the products that could be formed as confirmed by testing with ^{14}C substrates. Paper chromatography using doubly labeled (^{13}N and ^{14}C) products and ninhydrin staining have also been performed on selected runs and have also aided in establishing the radiochemical purity of the L-alanine (*8*).

Similarly, the short half-life of ^{13}N does not permit the performance of USP pyrogen and sterility tests prior to use. The terminal Millipore filtration removes bacteria and presumably renders the solution sterile. Sterile and pyrogen-free reagents and glassware are utilized. The Limulus test for endotoxin is performed on the buffer solution from the column containing the silica beads with immobilized enzymes prior to each use because this appears to be the biggest potential source of pyrogen in the system. Aliquots of the final ^{13}N-L-alanine solution are checked after the fact using USP sterility and pyrogen tests.

A method is outlined for the production of ^{13}N-labeled L-amino acids of radiopharmaceutical purity. This involves synthesis using enzymes which have been immobilized on a solid-state support.

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