^{13}N-Labeled L-Amino Acids for in Vivo Assessment of Local Myocardial Metabolism[1a]

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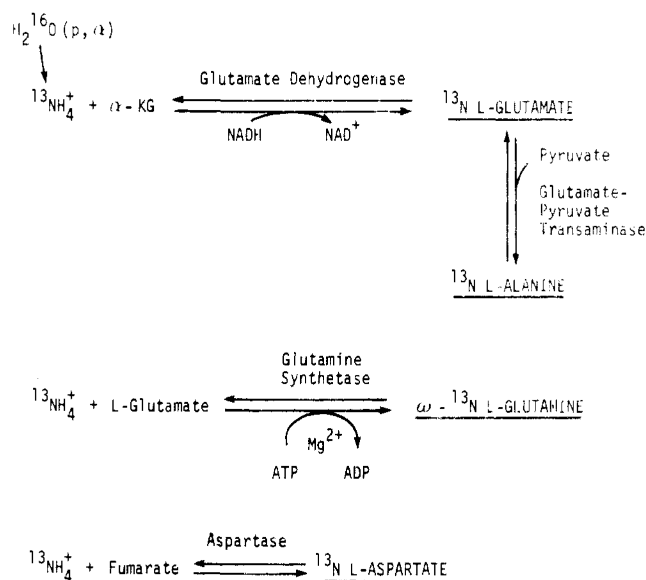
The hot cell synthesis of sterile, pyrogen-free ^{13}N-labeled L-amino acids was accomplished by employing the appropriate immobilized enzymes on a CNBr-activated Sepharose support and using remote, semiautomated systems. The syntheses were completed 6-12 min after cyclotron production of [^{13}N]ammonia. Myocardial time-activity curves after intracoronary injection of ^{13}N-labeled L-amino acids in dogs were triexponential in both normal and ischemic myocardium. Higher retention of ^{13}N activity was observed in ischemic segments. Positron computed tomography imaging also showed increased uptake of ^{13}N-labeled L-glutamate and L-alanine in ischemic segments compared with normal myocardium when blood flow corrections were made. Myocardial transaminases are primarily responsible for the observed retention fractions. It suggests the participation of the carbon skeletons of these amino acids in the Krebs cycle.

The assembly of accurate information with respect to the operation of enzymes involved in nitrogen metabolism in the heart from in vivo studies in animals or humans is at a primitive stage. Most experimental information on myocardial substrate metabolism has been derived from the isolated rat and rabbit heart and open-chest, in situ dog and pig heart models.[2-7] Evidence of the key role played by amino acids in the myocardium comes from the observation that both patients with chronic ischemic heart disease[8] and animals with induced congestive heart failure exhibit specific alterations in myocardial amino acids metabolism. Equally important is the possible role of amino acids in anaerobic pathways of energy production.[9-11] In this investigation we describe the remote controlled, semiautomated enzymatic synthesis of several L-amino acids labeled with ^{13}N (half-life 9.96 min) using immobilized enzymes and their first application for in vivo assessment of local myocardial metabolism.

Enzymatic Syntheses. Substitution of ^{13}N, a positron-emitting radioisotope, for ^{14}N in L-amino acids would render agents that are indistinguishable physiologically and chemically from their natural counterparts. ^{13}N-Labeled L-amino acids have previously been synthetized enzymatically,[12-17] but only a few of them have been prepared using immobilized enzymes.[18-20] Immobilization of glutamate dehydrogenase[21] has permitted synthesis of pyrogen-free ^{13}N-labeled L-glutamate suitable for imaging osteogenic sarcoma, brain tumors, and the heart in humans.[19,22] Scheme I describes the enzymatic reactions used to produce the respective amino acids.

Scheme I



[^{13}N]Ammonia was produced by our biomedical cyclotron (CS-22 Cyclotron Corp.) using the $^{16}O(p,\alpha)^{13}N$ reaction on H_2O.[23] Gaseous [^{13}N]ammonia was swept with N_2

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Table I

^{13}N-labeled amino acid	yields[a]	radiochem. purity, %	residue fraction, %		$T_{1/2}$, min	
			control[b]	ischemia[b]	control[b]	ischemia[b]
L-glutamate	33	>99	8.1 ± 3.1 (2)	11.5 ± 3.2 (2)	114.1 ± 26.5 (4)	81.2 ± 6.1 (2)
L-glutamine[c]	15	>99	17.3 ± 6.1 (3)	20.5 ± 3.5 (3)	15.9 ± 0.2 (3)	17.5 ± 2.0 (3)
L-alanine	8	>95	18.5 ± 2.1 (2)	19.5 ± 1.0 (2)	88.0 ± 2.0 (2)	77.5 ± 3.5 (2)
L-aspartate	1	>99	14.0 ± 4.2 (2)	17.0 ± 5.7 (2)	147.0 ± 12.0 (2)	172.0 ± 34.0 (2)

[a] Yields are not decay corrected. They are indicated as percentages of the total ^{13}N activity trapped (typically 175-200 mCi) when $^{13}NH_3$ is bubbled through the solution containing the appropriate substrates. [b] Average values ± 1 standard deviation. Number of experimental runs in parentheses. [c] ω-^{13}N labeled.

into a 5-mL buffered substrate-containing solution which was then transferred to the appropriate enzyme column(s). In the case of L-[^{13}N]glutamate, L-[ω-^{13}N]glutamine, and L-[^{13}N]aspartate, a final AG 50W-X12 cation-exchange column was used to remove unreacted [^{13}N]ammonia. The enzyme column for producing L-[^{13}N]alanine consisted of a top layer of immobilized glutamate dehydrogenase and a bottom layer of immobilized glutamate–pyruvate transaminase. An AG 1-X4 anion-exchange column was used to trap unreacted L-[^{13}N]glutamate while, again, a cation exchange column removed unreacted [^{13}N]ammonia. In all cases, final sterilization was accomplished by Millipore filtration.[24] The enzymatic syntheses were performed by remote, semiautomated control of the chemical unit operations as described in a preliminary report by Barrio et al.[25] Typically, the syntheses were completed 6–12 min after cyclotron production of [^{13}N]ammonia. The immobilized enzymes are quite stable, protein loss is minimal, and the use of 2 M KCl as a preservative has proven to be an excellent inhibitor of bacterial growth in the column. The columns are reusable for several months without significant loss of enzymatic activity. Table I lists actual yields and percent conversions for each ^{13}N-labeled L-amino acid.

Rapid verification of amino acid purity was routinely achieved by adapting the method of *o*-phthaldialdehyde (OPT) precolumn fluorescence derivatization.[26] The strongly fluorescent amino acid–OPT complex (isoindigo derivative, λ_{exc} 340 nm; λ_{em} 455 nm)[27] can be separated with reversed-phase HPLC and detected to subpicomole levels (Ultrasphere ODS, 5 μm, 4.6 × 150 mm i.d., 55% 100 mM potassium phosphate buffer, pH 7.4, and 45% MeOH; flow rate, 1.0 mL/min; fluorescence detector). Coupling a radioactivity detector to the system permitted simultaneous analysis of radiochemical purity (Table I).

Biological Results

Two different techniques were used in this work for the in vivo investigation of amino acid metabolism in myocardium: (a) intracoronary bolus injection and kinetic analysis of the tissue uptake using external radiation detectors and (b) intravenous administration and tomographic imaging. Myocardial time–activity curves after intracoronary injections of ^{13}N-labeled L-amino acids in dogs (see Experimental Section) were triexponential in normal and ischemic myocardium. They revealed a short half-time for the vascular phase (4–8 s), a rapid second phase (20–60 s), and a slower third phase (15–170 min). That transamination reactions are primarily responsible for the radioactivity retained by the tissue (residue fraction) is demonstrated by the fact that inhibition of myocardial transaminase activity with aminooxyacetic acid[11] decreased the tissue clearance half-time of L-[^{13}N]glutamate, L-[^{13}N]aspartate and L-[^{13}N]alanine with no effect on L-[ω-^{13}N]glutamine clearance. As an example, the residue fraction half-time of L-[^{13}N]glutamate decreased from 124.4 to 38.8 min in two experimental runs upon enzymatic inhibition with aminooxyacetic acid. For all compounds tested the residue fractions were 0.1–0.2 in normal myocardium and, slightly, 0.02–0.07, but consistently higher during ischemia. Positron-computed tomography (PCT) imaging of the dog heart[28] showed an apparent unchanged myocardial uptake of L-glutamate and L-alanine in ischemic segments compared to normal myocardium,[29] despite reduced blood flow as demonstrated by [^{13}N]ammonia PCT imaging. When blood flow corrections are made, the ^{13}N activities retained in ischemic segments are higher than that observed in normal myocardium. This ^{13}N activity reflects a residue fraction incorporated into nitrogen pools after, primarily, enzymatic transamination of the ^{13}N-labeled amino acids. The role of transamination reactions in tissue retention of the ^{13}N activity indicates that the carbon skeletons of these amino acids are involved in energy-producing schemes in the heart, namely, entrance into the Krebs cycle as α-keto acids. Because of the relatively increased tracer concentration during ischemia and the relationship of the amino acids to the Krebs cycle, ^{13}N-labeled L-amino acids could be of value for in vivo studies of energy metabolism in normal and ischemic myocardium.

Experimental Section

Enzymes were obtained from Sigma Chemical Co. Substrates were either from Sigma Chemical Co. or Calbiochem-Behring Corp. Cyanogen bromide activated Sepharose was from Pharmacia; columns and ion-exchange resins were from Bio-Rad Laboratories.

Enzyme Immobilization. Bovine liver L-glutamic acid dehydrogenase (EC 1.4.1.3) (500 units), sheep brain L-glutamine synthetase (EC 6.3.1.2) (50 units),[30] porcine heart L-glutamic–pyruvic acid transaminase (EC 2.6.1.2) (400 units), and *Bacillus cadaveris* L-aspartase (EC 4.3.1.1) (50 units) were each coupled

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(29) After ^{13}N-labeled L-amino acid injection, the normal dog myocardium is poorly visualized. Tissues with relatively high amino acid uptake are spinal bone marrow, salivary glands, liver, gut, and pancreas. Species differences greatly influence myocardial extraction of amino acids (Gelbard, A. S.; MacDonald, J. M.; Reiman, R. E.; Laughlin, S. *J. Nucl. Med.* **1975**, *16*, 529).
(30) Although glutamine synthetase (Rasulov, A. S.; Evstigneeva, Z. G. *Prikl. Biokhim. Mikrobiol.* **1978**, *14*, 150) and L-aspartase (Tosa, T.; Sato, T.; Mori, T.; Matuo, Y.; Chibato, I. *Biotechnol. Bioeng.* **1973**, *25*, P69) have been immobilized, neither L-[^{13}N]glutamine nor L-[^{13}N]aspartate have been reported synthesized in this manner.

to 500 mg of CNBr-activated Sepharose in the presence of 30 mM sodium phosphate/0.5 M NaCl, pH 8.3. Previous to this process all enzymes were dialyzed against 30 mM sodium phosphate, pH 7.5. The Sepharose–enzyme mixture (total volume 10–15 mL) was gently agitated and permitted to react over a period of 2 h at room temperature. Residual active groups on the Sepharose support were hydrolyzed by passage of 30 mM Tris-HCl, pH 8.0, through the column for 2 h at room temperature. The column was next washed alternately with 30 mM sodium phosphate at high (8.3) and low (4.0) pH, which removed any protein ionically bound to the Sepharose support. The column was stored at 4 °C in 2 M KCl/30 mM sodium phosphate, pH 7.5.

Enzymatic Syntheses. Gaseous [^{13}N]ammonia (175–200 mCi) produced by the $^{16}O(p,\alpha)^{13}N$ reaction on H_2O[23] was swept with N_2 into a 5-mL buffered solution containing the following substrates: for the synthesis of L-[^{13}N]glutamate, 30 mM sodium phosphate, pH 7.5, 1.6 mM α-ketoglutarate, 1 mM NADH; for L-[^{13}N]alanine, 30 mM sodium phosphate, pH 7.5, 0.8 mM α-ketoglutarate, 1 mM NADH, 5 mM pyruvic acid; for L-[^{13}N]-glutamine, 30 mM sodium phosphate, pH 7.5, 20 mM $MgCl_2$, 6 mM L-glutamate, 6.8 mM ATP; for L-[^{13}N]aspartate, 30 mM sodium phosphate, pH 7.5, 20 mM $MgCl_2$, 25 mM fumarate.

For the synthesis of ^{13}N-labeled L-glutamate, the glutamate dehydrogenase column was used. The buffered substrates with [^{13}N]ammonia were allowed to pass by gravity through the column, and the column was subsequently washed with 3 mL of 30 mM sodium phosphate buffer, pH 7.5, and all elutions were combined. A similar procedure was followed for L-[^{13}N]glutamine and L-[^{13}N]aspartate using the corresponding enzyme columns. For L-[^{13}N]alanine, the glutamate dehydrogenase/glutamate–pyruvate transaminase column was used. Five milliliters of 30 mM sodium phosphate, pH 7.5, was used to wash the column after passage of the substrate containing solution. A final AG 50W-X12 cation-exchange resin (0.7 × 7.5 cm; Na form) equilibrated with 30 mM sodium phosphate, pH 7.5, was used to retain any unreacted [^{13}N]ammonia in the synthesis of L-[^{13}N]glutamate, L-[^{13}N]alanine, L-[^{13}N]aspartate, and L-[^{13}N]glutamine. The ^{13}N-substituted L-amino acid solution was made isotonic. Finally, a 0.22-μm pore size filter was used to sterilize the product as it was passed into a sterile, pyrogen-free vial.

Myocardial Residue Fraction of ^{13}N-Labeled Amino Acids. The myocardial tracer residue fraction was studied with a single pass uptake technique in open-chest instrumented dogs. Experimental protocol, animal instrumentation, and data collection and processing used in our laboratory have been reported in detail previously.[31] Briefly, the initial capillary transit and the retained amount of ^{13}N were recorded, and a time–activity curve was obtained with a scintillation detector following bolus injection of 10–20 μCi of the ^{13}N-labeled amino acids into the left circumflex coronary artery. Data were collected in 0.1-s increments for 20 min and stored in a digital computer. Experimental runs were performed at control state and during ischemia. Ischemia was induced by mechanical occlusion and flow reduction, 20 min prior to injection and during data acquisition. Computer-assisted curve fitting revealed a triexponential time–activity curve. The residue fraction of ^{13}N retained in myocardium was determined with a graphic extrapolation of the third slow clearance phase (C) back to the time of the maximal peak (A) representing the total amount of activity injected. The residue fraction was computed as the ratio of C/A. The half-times of these residue fractions were calculated from the slope of the third slow clearance phase ($T_{1/2}$, Table I). These methods have been previously employed and validated for studies in the brain[32,33] and in the heart.[31,34,35] Inhibition of myocardial transaminases with aminooxyacetic acid was produced as previously described.[11]

Tomographic Imaging. Cross sectional images were obtained with the UCLA positron emission computed axial tomograph, ECAT, as described in detail previously.[36] Again, open-chest instrumented dogs were studied with ischemia induced by mechanical occlusion and flow reduction with a screw-type occluder placed around the left anterior descending coronary artery. Flow reduction in the antero-septal myocardium was documented by tomographic imaging using 10–20 mCi of [^{13}N]ammonia injected intravenously.[31,36] Then, imaging was performed under the same conditions, but 10–20 mCi of ^{13}N-labeled amino acid was given intravenously. Imaging was begun 5 min after injection. The residual of tracer was quantitated by assigning regions of interest to normal and ischemic myocardial segments, and their ratios were calculated for [^{13}N]ammonia (F), indicating the degree of flow reduction, as well as for ^{13}N-labeled amino acids (AA). The ratios AA/F allowed to estimate the amount of ^{13}N activity remaining in ischemic myocardium in relation to flow reduction. These ratios were consistently higher than 1.

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Benz-Fused Mesoionic Xanthine Analogues as Inhibitors of Cyclic-AMP Phosphodiesterase

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Mesoionic xanthine analogues, such as derivatives of mesoionic thiazolo[3,2-*a*]pyrimidine, constitute a new class of inhibitors of adenosine cyclic 3′,5′-monophosphate (cyclic-AMP) phosphodiesterase (PDE). A series of 16 benz-fused analogues of the mesoionic thiazolopyrimidines were prepared and found to be more active than their nonfused counterparts, when assayed using bovine heart PDE.

Adenosine cyclic 3′,5′-monophosphate (cyclic-AMP) phosphodiesterase (PDE) is an enzyme which converts cyclic AMP to adenosine 5′-monophosphate and is responsible, in part, for regulation of intracellular levels of this cyclic nucleotide. Methylated xanthines, such as theophylline, inhibit cyclic nucleotide PDE, and, in an earlier publication, we reported that mesoionic xanthine

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