BIOSYNTHETIC METHODS FOR INCORPORATING POSITRON-EMITTING RADIONUCLIDES INTO COMPOUNDS OF BIOMEDICAL INTEREST

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SUMMARY

The progress that has been made in incorporating the short-lived, positron-emitting isotopes N-13 and C-11, into biologically active compounds by biosynthetic means is reviewed. These methods include the use of whole cells or plant leaves to carry out a sequence of metabolic reactions, such as nitrogen fixation or photosynthesis, and the use of purified enzymes to catalyze specific chemical reactions, to form the labeled compounds.

Key Words: Biosynthesis, Enzymes, Nitrogen-13, Carbon-11, Positrons.

INTRODUCTION

The early work with the short lived positron emitting nuclides nitrogen-13 ($t_{1/2}$=10 min.) and carbon-11 ($t_{1/2}$=20 min.) was concerned with their use as tracers in biosynthetic and metabolic studies. These nuclides were used for studies in the assimilation of nitrogen and carbon dioxide in plants and bacteria as well as for the synthesis of some simple compounds. Such organic acids as acetic, lactic, propionic, succinic

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and fumaric were labeled by organic synthetic procedures with C-11 in the carboxyl position, and methane and acetic and propionic acids were labeled with C-11 by biosynthetic methods using microorganisms. For reviews covering the early work with carbon-11 see Kamen (1) and Buchanan and Hastings (2); the first paper utilizing N-13 involving nitrogen fixation was by Ruben et al. (3). The short half-lives of these two nuclides and the requirement that they must be produced by an accelerator such as a cyclotron limited their use. The subsequent discovery of reactor-produced long-lived C-14 made this the preferred nuclide for studying intermediary metabolism, and interest in the short-lived nuclides declined during the 1950's and early 1960's.

Renewed interest in the synthesis and use of compounds labeled with short-lived positron-emitting isotopes came about as a result of the greater availability of accelerators to biomedical investigators, who recognized the utility of labeling metabolites with radionuclides of their natural elements, and of the development of imaging instruments using annihilation coincidence detecting techniques to determine the biodistribution of these labeled compounds from outside the body. Indeed, the short half-lives of N-13 and C-11 would now prove advantageous for clinical studies because of the limited radiation burden imposed on the patient. The development of positron emission tomography, which quantitatively determines tissue radioactivity concentrations, has placed compounds labeled with positron-emitting isotopes in a unique position. An increasing number of suitably labeled metabolites will be required to study specific physiological processes. Although most of the advances in the recent development of compounds labeled with positron emitters has been by organic synthetic

procedures (see reviews by Wolf and Redvanly (4), and Wolf and Fowler (5)), there have been a number of reports concerned with the biosynthesis of positron-labeled compounds. The present review will discuss this work. These studies will be divided into those syntheses carried out by whole organisms, and those involving one or more specific chemical reactions which are enzyme-catalyzed. In the former cases, the organisms are exposed to a simple labeled substrate under a particular set of conditions for a short period of time and are allowed to synthesize a number of labeled metabolites. The labeled products are separated from the cells and then may be further purified by such methods as high pressure liquid chromatography. In enzymatic syntheses, the number of labeled compounds that have to be separated is limited and may be carried out by ion exchange chromatography.

When compared to organic synthetic procedures, enzymatic methods have the advantage of being rapid and specific and preclude the problems of formation of racemic mixtures. The commercial availability of specific enzymes is limited, however, and enzyme purification procedures are time consuming. Furthermore, if the enzymatic labeling method is carried out in a batch process, it is necessary to replenish the enzyme after each synthesis. With the batch method, it is also difficult to prepare a labeled product suitable for clinical use because of the difficulty of removing the potentially pyrogenic and antigenic enzyme protein from the labeled product. However, the enzyme can be immobilized onto a solid support and the substrates and cofactors can react as they are passed through the bound enzyme. In this manner the labeled product is free of enzyme and the immobilized enzyme column can be used repeatedly for

synthetic studies. Although biosynthetic studies, using bacterial or plant cells to label metabolites have been carried out only by batch methods, techniques are available to immobilize cells onto solid-state supports. It should be feasible, then, to prepare labeled compounds by biosynthetic methods, using whole cells, that would be suitable for clinical studies.

NITROGEN-13

Biosynthetic methods, using whole organisms, have not been used for the production of N-13 labeled compounds. N-13 in the form of (N-13) N_2 and (N-13) ammonia has been used to study nitrogen metabolism and fixation in plants and microorganisms. In these studies the N-13 label was found to be more useful than the stable isotope N-15 for detecting and quantifying small amounts of nitrogen that were fixed within seconds after incubation. Wolk and co-workers (6-8) have studied the pathways of assimilation of (N-13) N_2 and (N-13) ammonia in symbiotic and free-living prokaryotes by using electrophoresis to determine the metabolic fate of the labeled nitrogen and ammonia and by using specific enzyme inhibitors to discern their effects on the products of fixation. The N-13 activity distribution in the labeled amino acids indicated that the initial pathway of metabolism of N_2-derived nitrogen in a number of species of prokaryotes is by the glutamine synthetase/glutamate synthase reactions.

The initial studies on the enzymatic labeling of L-(N-13) amino acids (9-12) were based on producing (N-13) ammonia in solution as the precursor by the ^{12}C (d,n)^{13}N reaction on methane (13). However, the

formation of impurities and of carrier ammonia resulting from the bombardment of methane limited the yields of labeled amino acids that could be synthesized from labeled ammonia produced in this manner. (N-13) Ammonia of higher purity and specific activity has been obtained by the $^{16}O\ (p,\alpha)^{13}N$ reaction on water to produce labeled nitrates and nitrites which can be reduced to (N-13) ammonia with titanous hydroxide (14) or Devarda's alloy (15,16). Several amino acids have been synthesized by the direct introduction of the $^{13}NH_2$ moiety into the amine or amide position of the appropriate precursor. L-(N-13) Glutamate has been prepared in yields of 75-95% by the reductive amination of α-ketoglutarate in a reaction catalyzed by glutamate dehydrogenase in the presence of the coenzyme reduced nicotinamide adenine dinucleotide (NADH) (17,18). Labeled glutamate was separated and purified by passage through a cation exchange column. The commercial source of the enzyme is beef liver and it has been immobilized on porous derivatized (N-hydroxysuccinimide) silica beads (19) or CNBr activated Sepharose (20). Glutamate dehydrogenase has a broad substrate specificity and has been used to label L-(N-13) valine from α-ketoisovaleric acid, L-(N-13) alanine from pyruvic acid and L-(N-13) leucine from α-ketoisocaproic acid in yields varying from 7-26% using the batch method (12). In our laboratory glutamate dehydrogenase immobilized on CNBr activated Sepharose has been used to label valine, leucine, methionine and α-amino butyric acid in yields of up to 10% using the appropriate α-keto acid.

L-(N-13) Glutamine, labeled in the amide position, has been prepared in high yield in a reaction catalyzed by glutamine synthetase (11,17). The incubation mixture contained, in addition to (N-13) ammonia

and enzyme, L-glutamic acid, adenosine triphosphate, Mg^{++}, and 2-mercapto-ethanol. The source of the commercial enzyme is sheep brain. The enzyme is not stable in solution and has not yet been immobilized. Thus synthetic reactions are carried out by the batch method.

L-(N-13) Asparagine, labeled in the amide position, has been synthesized in a reaction catalyzed by asparagine synthetase using enzymes purified from extracts of Novikoff hepatoma (15) or Escherichia coli (21). (N-13) Ammonia, enzyme, L-aspartic acid, adenosine triphosphate and Mg^{++} were components of the reaction mixture. Radiochemical yields were low with a conversion of 10-20% after a 10 min incubation. The enzyme is not available commercially and has not been immobilized.

Although a number of L-(N-13) amino acids besides L-(N-13) glutamate have been labeled by the reductive amination of the corresponding α-keto acids in reactions catalyzed by glutamate dehydrogenase, the yields have been low (12). A preferred way to synthesize the other N-13 amino acids is to synthesize L-(N-13) glutamate first, and then enzymatically transaminate the appropriate α-keto acid with the labeled amino group of glutamate in reactions catalyzed by glutamate α-keto acid transaminases. Cohen et al. prepared L-(N-13) alanine in this manner (19,22). Glutamate dehydrogenase and glutamate-pyruvate transaminase were immobilized onto derivatized silica beads and the formed L-(N-13) alanine was separated from (N-13) ammonia and L-(N-13) glutamate by ion exchange chromatography. L-(N-13) Aspartic acid, L-(N-13) tyrosine and L-(N-13) phenylalanine have been labeled by the synthesis of L-(N-13) glutamate followed by the transamination of oxaloacetate, p-hydroxyl phenylpyruvate or phenylpyruvate, respectively, in reactions catalyzed by a commercial source of pig heart

glutamate-oxaloacetate transaminase (23). Analyses of the reaction mixture by high pressure liquid chromatography demonstrated that 85% of the labeled amino group was transferred from glutamate to form L-(N-13) aspartate; 40% to form L-(N-13) tyrosine; and 15% to form L-(N-13) phenylalanine after a 5 minute incubation with the glutamate-oxaloacetate transaminase reaction mixture and the appropriate α-keto acid. The α-keto acids of leucine and dihydroxylphenylalanine but not of valine, tryptophan or asparagine could also act as amino acceptors in reactions catalyzed by the enzyme preparation.

CARBON-11

Biosynthetic labeling has been carried out with plant leaves or plant cells using (C-11) carbon dioxide produced by the $^{14}N\ (p,\alpha)^{11}C$ reaction on nitrogen gas as the labeled precursor. A mixture of (C-11) glucose and (C-11) fructose has been prepared by photosynthesis using light-starved Swiss chard (24,25) or Broad Bean (26) leaves. Improved yield of labeled glucose and separation of the products by high pressure liquid chromatography has been reported (27). Marine algae have been used to synthesize (C-11) mannitol, (C-11) galactose and (C-11) glycerol (28).

A number of compounds, labeled with C-11, have been prepared by enzymatic synthesis. In all publications the enzymatic procedures have been by batch methods.

L-(C-11) Aspartic acid, labeled in the carboxyl position, was synthesized from $^{11}CO_2$ by Hara et al. in a series of enzymatic steps (29). First $^{11}CO_2$ was converted to $H^{11}CO_3^-$ by the reaction of carbonic anhydrase.

Oxaloacetate was then labeled with C-11 by the catalytic action of oxaloacetate decarboxylase and then transaminated to L-(C-11) aspartic acid by glutamate-oxaloacetate transaminase. A crude preparation of chicken liver acetone powder was the source of oxaloacetate decarboxylase and glutamate-oxaloacetate transaminase. The radiochemical yield was about six per cent which may be increased by the use of more highly purified enzymes.

Spolter et al. have synthesized several C-11 compounds associated with the tricarboxylic acid cycle. Their strategy was to label the key intermediate acetyl CoA and utilize this compound to tag other metabolites. To do this (C-11) acetic acid was prepared by reacting $^{11}CO_2$ with methyl $MgCl_2$. The labeled acetate was then incubated with adenosine triphosphate and $MgCl_2$ in a reaction catalyzed by acetate kinase to yield acetyl phosphate (30). This was then converted to (C-11) acetyl CoA and finally (C-11) citric acid in the presence of phosphotransacetylase and citrate synthase (31). (C-11) Citric acid was prepared by an alternate method which involved reacting $^{11}CO_2$ with phosphoenolpyruvate to form (C-11) oxaloacetate followed by reaction with acetyl CoA to yield (C-11) citric acid in the presence of phosphoenolpyruvate carboxylase and citrate synthase, respectively. (C-11) pyruvate was also synthesized by reacting $^{11}CO_2$ and acetyl CoA in the presence of pyruvate-ferridoxin oxidoreductase extracted from Clostridium acidi urici. The synthesized (C-11) pyruvate was then converted to (C-11) lactic acid with lactic dehydrogenase. The labeled lactic acid was purified by ion exchange chromatography and a yield of three - five per cent was obtained (32). (C-11) Pyruvate may also be converted to L-(C-11) alanine by the enzyme

glutamate-pyruvate transaminase. Acetyl carnitine, associated with fatty acid transport to myocardial mitochondria, was labeled by reacting (C-11) acetyl CoA with carnitine in the presence of carnitine acetyltransferase (33).

Hippuric acid, a compound useful for quantitation of renal tubular secretion, was labeled with C-11 by reacting (C-11) benzoic acid, prepared by carbonating phenylmagnesium bromide with $^{11}CO_2$, and glycine. Rat liver mitochondria, containing benzoyl CoA synthetase and glycine acyltransferase, catalyzed the reactions (34).

Thymidine and thymidylic acid have been labeled with C-11 in reactions involving enzymes of folate metabolism (35). $^{11}CO_2$ was reduced to (C-11) formaldehyde with lithium aluminum hydride. The labeled formaldehyde served as a one-carbon donor by reacting nonenzymatically with tetrahydrofolate to form N^5, N^{10}-(^{11}C-methylene) tetrahydrofolate. Deoxyuridylate was then converted to (C-11)-methyl-labeled deoxythymidylate in a reaction catalyzed by thymidylate synthetase, which had been purified and crystallized from Lactobacillus casei. Enzymatic treatment of the labeled nucleotide with alkaline phosphatase yielded (C-11) thymidine. The preparation of (C-11) thymidine by the batch method took 110 minutes. Thymidylate synthetase has since been immobilized on arylamine-glass beads by the authors (36) and thus could be prepared by this method.

Enzymes have been used to resolve racemic mixtures of amino acids that have been labeled with C-11 by organic methods. DL-(C-11) phenylalanine, prepared from (C-11) cyanide by a modified Bucherer-Strecker reaction, has been resolved into its D- and L- isomers by the action on the racemate of immobilized L- and D- amino acid

oxidases, respectively (37). After incubation with the appropriate amino acid oxidase, either optically active isomer of phenylalanine was separated from phenylpyruvic acid by cation exchange chromatography.

CONCLUSIONS

During the past decade, a number of compounds labeled with short-lived positron emitting nuclides have been synthesized. Although most of the compounds were prepared by organic chemical procedures, there have been a significant number of studies utilizing biosynthetic methods to label compounds with N-13 and C-11. In most instances, experiments have been limited to demonstrating the feasibility of these biosynthetic procedures for introducing the label into complex molecules. Future emphasis will be based on the preparation of labeled compounds, suitable for clinical studies, utilizing immobilized cells or enzymes.

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