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ENZYMATIC SYNTHESIS OF CARBON-11 ACETYL COENZYME A

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SUMMARY

An enzymatic synthesis of [^{11}C]acetyl coenzyme A from [1-^{11}C]acetate is described. $^{11}CO_2$ was reacted with methylmagnesium bromide in diethyl ether at 0 °C. The excess of Grignard reagent was hydrolyzed by adding methanol. After evaporation of the solvents, the residue was redissolved in hydrochloric acid and [1-^{11}C]acetic acid was removed by acid distillation. In the presence of cofactors the [1-^{11}C]-acetic acid was sent over an enzyme reactor containing immobilized acetyl coenzyme A synthetase (Acetate : CoA ligase (AMP forming) ; EC 6.2.1.1).

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About 71 % of $^{11}CO_2$ was converted into [^{11}C]acetyl coenzyme A, yielding 250 mCi (9.2 GBq) with a specific activity of 390 mCi/μmol (14.4 GBq/μmol) at EOB + 22 min. It represents a sufficient amount of labelled precursor to allow syntheses of other ^{11}C-labelled compounds.

Key Words : Carbon-11 acetyl coenzyme A, acetyl coenzyme A synthetase, enzyme reactor, [^{11}C]precursor.

INTRODUCTION

Carbon-11 acetyl coenzyme A is a new precursor designed for the preparation of labelled compounds, useful for non-invasive biomedical studies with positron emission tomography (PET). The molecule will be of great biomedical interest, since it serves as a cofactor for numerous enzymes.

Carbon-11 acetyl coenzyme A, labelled in the carbonyl function of the acetyl group, can be prepared from [1-^{11}C]-acetate by two subsequent enzyme-catalyzed reactions e.g. acetate kinase (EC 2.7.2.1) and phosphotransacetylase (E.C. 2.3.1.8) or by a one-step enzymatic reaction with acetyl coenzyme A synthetase (EC 6.2.1.1). We selected the second possibility (Fig. 1) because the affinity of acetyl coenzyme A synthetase for acetate is much higher than of acetate kinase.

$$^{14}N(p,\alpha)^{11}C \xrightarrow{\text{traces } O_2} {}^{11}CO_2$$

$$^{11}CO_2 + CH_3MgBr \xrightarrow[\text{2. methanol}\\\text{3. HCl}]{\text{1. diethyl ether 0 °C}} CH_3{}^{11}COOH$$

$$CH_3{}^{11}COOH + ATP + CoA\text{-}SH \xrightarrow[Mg^{2+}\ (EC\ 6.2.1.1)]{\text{Immobilized acetyl CoA synthetase}} CoA\text{-}S\text{-}{}^{11}COCH_3 + AMP + PPi$$

Fig. 1. Scheme for the synthesis of [^{11}C]acetyl coenzyme A.

Because carbon-11 is a positron emitter with a half-life of 20.4 min, a rapid synthesis is needed. The enzyme is immobilized to obtain a labelled end-product which is free of enzyme. The chosen carrier, controlled pore glass, is mechanically stable permitting high flow rates. This is an important advantage in the synthesis of short-lived radiopharmaceuticals.

EXPERIMENTAL

Materials

Acetyl coenzyme A synthetase (ACS) from baker's yeast, trilithium coenzyme A (CoA), disodium adenosine-5'-triphosphate (ATP) and trilithium acetyl coenzyme A were obtained from Sigma Chemical Company (USA). Controlled pore glass beads (CPG 125-180 µm) with a pore size of 50 nm were purchased from Serva Feinbiochemica GmbH. Other products were p.a grade from Merck (FRG) or Janssen Chimica (Belgium).

Enzyme assay and unit definition

The enzyme activity in solution was assayed as described by Jones et al. (1). Optimization of the reaction parameters led to the following incubation mixture : one ml contained an appropriate amount of enzyme (0.02-0.04 U), 100 µmol potassium acetate, 10 µmol ATP, 0.25 µmol CoA, 5 µmol $MgCl_2$ and 200 µmol hydroxylaminehydrochloride, the latter previously neutralized to pH 7.4 with KOH. After incubation for 20 min at 37 °C, 2.0 ml of a ferric chloride solution (0.37 M $FeCl_3$, 0.2 M trichloroacetic acid and 0.66 M HCl) were added.

For the enzyme immobilized on CNBr activated CPG, the final incubation mixture contained 50 mg enzyme-loaded glass beads and one ml of a solution containing 25 µmol potassium

phosphate pH 7.4, 10 µmol potassium acetate, 10 µmol ATP, 0.5 µmol CoA, 20 µmol $MgCl_2$ and 200 µmol neutralized hydroxylamine. The enzyme-loaded glass beads were shaken for 20 min at 40°C. After reaction, 800 µl of the supernatant was mixed with 2.0 ml of the ferric chloride solution.

Absorbance was measured at 500 nm against a blank containing 2.0 ml of the ferric chloride solution and 1.0 ml of a mixture of 100 µmol potassium phosphate pH 7.4 and 200 µmol neutralized hydroxylamine.

One unit of ACS is defined as that amount of ACS which catalyzes the formation of one micromol acetyl coenzyme A per minute under the described conditions.

Preparation of the enzyme reactor

Acetyl coenzyme A synthetase was bound onto CNBr activated CPG beads (CPG 500, 125-180 µm). One gram CPG was CNBr activated according to the method of Weetall (2).

Immediately after preparation, the activated glass beads were rinsed with ice-cold water, divided into 100 mg portions and each treated at 0 °C with 2.4 units ACS dissolved in 50 µl preservative buffer (0.5 M potassium phosphate pH 7.4, 10 mM glutathione and 0.5 mM EDTA) and 100 µl 0.6 M potassium phosphate pH 7.2. Two hours of shaking at 4 °C completed the immobilization. After pooling the portions, unbound protein was removed by rinsing the enzyme-loaded glass beads with 0.05 M potassium phosphate pH 7.4 The enzyme-loaded beads were packed in a glass column (13.0 x 0.50 cm i.d.). The enzyme reactor was stored aseptically at 4 °C in preservative buffer. Before use, the column was placed in a water jacket at 37 °C and rinsed with 0.05 M potassium phosphate pH 7.4.

Set-up of the [^{11}C]acetyl CoA production (Fig. 2).

All parts of the set-up were connected by teflon tubing (0.8 mm i.d.). Electromagnetic valves (o) were used for remote-controlling of the system. $^{11}CO_2$ was produced by irradiation of nitrogen gas (10.5 bar) with 18 MeV protons at 15 µA for 20 min. The irradiated target gas was released to a hot-cell where $^{11}CO_2$ was frozen out into a stainless steel coil (A : 2 m x 2.1 mm i.d.) immersed in liquid nitrogen (flow rate 3 $l.min^{-1}$). The trapping of $^{11}CO_2$ took about 4 min.

The stainless steel coil with the trapped $^{11}CO_2$ was immersed in a water bath at room temperature. The $^{11}CO_2$ was swept out by a helium flow of 5.6 $ml.min^{-1}$ and sent to a reaction vessel (B : upper part 35 x 12 mm i.d. plugged with rubber septum, conical bottom 80 x 6 mm i.d.) containing 200 µl of 0.2 M methylmagnesium bromide. The vessel was previously cooled to 0 °C in an ice-water bath. The methylmagnesium bromide was prepared in diethyl ether according to Slegers et al (3).

After the trapping of $^{11}CO_2$, 100 µl of methanol was added to hydrolyze the excess methylmagnesium bromide. The mixture was evaporated to dryness under a helium flow by heating the reaction vessel (B) in an oil bath at 150 °C. Two hundred µl 0.25 M hydrochloric acid were added to dissolve the residue and the [^{11}C]acetic acid was distilled into a reaction vial (C : 5.0 ml volume, with conical bottom), previously filled with 800 µl of a solution containing 60 µmol potassium phosphate pH 7.6, 5 µmol CoA, 10 µmol ATP, 20 µmol magnesium chloride and 40 µmol potassium hydroxide. By peristaltic pumps (E and F) and a sample loop (G : 1.0.ml teflon loop, 221 x 2.4 mm i.d.) the content of vial C (final pH about 7.4) was sent at 1.0 $ml.min^{-1}$ over an acetyl CoA synthetase enzyme reactor at 37 °C (D : 13.0 x 0.50 cm i.d.).

The enzyme column was eluted with 0.05 M potassium phosphate pH 7.4. The column outlet was monitored with a G.M. tube and [^{11}C]acetyl CoA was collected in a final volume of 3.0 ml. The whole synthesis, starting from the trapping of $^{11}CO_2$ took about 22 min.

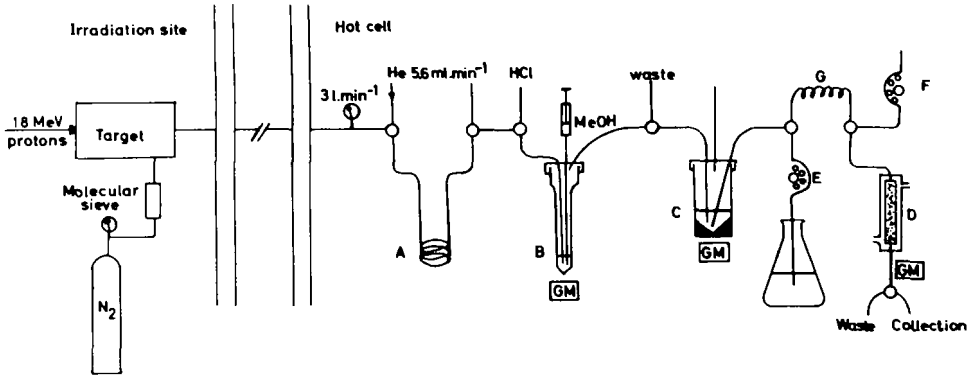

Fig. 2. Set-up for [^{11}C]acetyl coenzyme A synthesis.

The collected reactor eluate was submitted to radiochromatography by HPLC according to the chromatographic conditions used to assay enzymes involved in coenzyme A metabolism (4). Fifty µl were injected on a Spherisorb 5 ODS 2 column (150 x 3.2 mm). The column was eluted at 1.0 ml.min^{-1} with the mobile phase consisting of : aqueous phase ; methanol ; tetrahydrofurane : 410 ; 150 ; 4.5. The aqueous phase contains 5.10^{-2} M monosodium phosphate pH 7.0 and 10^{-2} M tetrabutylammonium hydrogen sulfate. The column temperature was set at 35 °C. Simultaneous UV detection at 258 nm and radioactivity detection (NaI/Tl) were applied.

RESULTS AND DISCUSSION

[1-^{11}C]acetic acid production

The target system for the production of $^{11}CO_2$ is described by Vandewalle and Vandecasteele (5). [1-^{11}C]acetic acid was produced by Grignard reaction of $^{11}CO_2$ with methylmagne-

sium bromide in diethyl ether at 0 °C. All connections and valves were thoroughly dried with dry helium. Any trace of water hydrolyzes the Grignard reagent resulting in ^{11}C-activity losses. A helium flow of 5.6 ml.min^{-1} was used to transfer $^{11}CO_2$ into reaction vessel B in order to allow a sufficient reaction time between $^{11}CO_2$ and the Grignard reagent. The ^{11}C-activity lost during the trapping of $^{11}CO_2$ in the Grignard reagent is also an indication of the quality of the latter (Table 1).

Table 1

Distribution and loss of [^{11}C]activity* (%)

untrapped $^{11}CO_2$ in the Grignard reagent (vessel B)	7.7 ± 7.4
^{11}C-loss after methanolysis and solvent evaporation	4.7 ± 1.9
remaining activity in vessel B after acid distillation	1.1 ± 0.6
ventilation outlet of buffer solution C	0.5 ± 0.4
enzyme reactor	0.3 ± 0.1
collection flask	85.7 ± 10.0

* Mean value ± s.d. for five productions

The Grignard reaction step represents the greatest loss of ^{11}C-activity. As the enzymatic conversion of [1-^{11}C]acetic acid is performed in aqueous solution, organic solvents must be removed to avoid any enzyme inhibition. Therefore the Grignard reaction was stopped by adding an excess of methanol immediately after the $^{11}CO_2$ trapping. Methanol was pre-

ferred instead of water because the methanol-diethyl ether mixture could be very rapidly evaporated without significant loss of ^{11}C-activity (Table 1).

After dissolving the Grignard residue with hydrochloric acid, [1-^{11}C]acetic acid and any trace of [^{11}C]acetone, which can be produced by subsequent Grignard reaction on $^{11}CO_2$, were distilled into reaction vial C. The hydrochloric acid amount was optimized to obtain a complete dissolution in reaction vessel B and a reproducible pH in vial C. The KOH in the buffer solution of vial C was necessary to neutralize the distilled acids and the enzyme cofactors. A final pH of about 7.4 was obtained which was the optimal pH for the enzymatic conversion (mean value ± s.d. for five productions: 7.35 ± 0.18).

[^{11}C]acetyl coenzyme A production

The mixture of [1-^{11}C]acetic acid, cofactors and buffer was pumped over an enzyme reactor containing immobilized acetyl coenzyme A synthetase.

The [^{11}C]acetyl coenzyme A peak was collected in a final volume of 3.0 ml with a pH of 7.4. The collected activity represents the highest percent of the total ^{11}C-activity (Table 1).

Results concerning the enzyme immobilization e.g. immobilization screening, immobilized enzyme stability, optimization of immobilization conditions, kinetic properties of the enzyme reactor for a high conversion of [1-^{11}C]acetate into [^{11}C]acetyl coenzyme A, are given elsewhere (6).

Radiochemical purity and specific activity of [^{11}C]acetyl coenzyme A

Radiochromatography of the reactor eluate was performed

as described in the experimental section. A typical chromatogram is given in Fig. 3. The [^{11}C]acetyl CoA peak represents about 82.8 % of the carbon-11 activity. From these data and the activity of the reactor eluate (Table 1), one can calculate that 71 % of $^{11}CO_2$ was converted into [^{11}C]acetyl CoA,

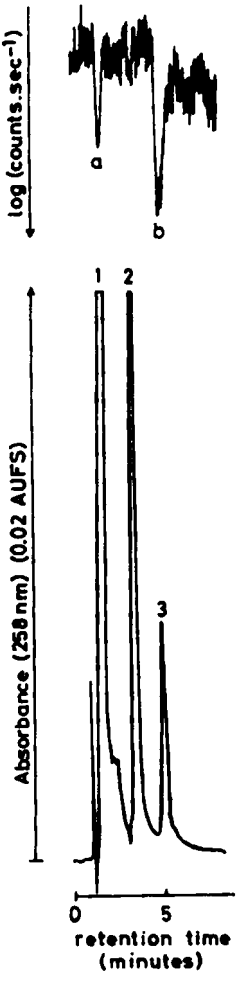

Fig. 3. Radiochromatography : UV detection at 258 nm : 1. adenosine phosphates (ATP,AMP) 2. CoA 3. acetyl CoA - Radioactivity detection (NaI/Tl) : a. [^{11}C]-acetate and [^{11}C]acetone b. [^{11}C]acetyl CoA.

yielding 250 mCi at EOB + 22 min. From the chromatographic data (Fig. 3) and a standard curve measured in the same experimental conditions, the carrier content was calculated as 0.65 µmol acetyl CoA (mean value of six productions), resulting in a specific activity of 390 $mCi.\mu mol^{-1}$ (EOB + 22 min).

The resulting [^{11}C]acetyl coenzyme A solution was not further purified to higher radiochemical purity, because for our purposes [^{11}C]acetyl coenzyme A is not an end-product but a precursor that will be used for subsequent enzymatic labelling of other molecules. However separation of [^{11}C]acetic acid and any trace of [^{11}C]acetone from [^{11}C]acetyl coenzyme A can easily be achieved by chromatography on a Dowex anion exchange resin (7).

In conclusion, a rapid high yielding enzymatic synthesis of [^{11}C]acetyl coenzyme A is described. After purification, [^{11}C]acetyl coenzyme A can be applied to study e.g. fatty acid metabolism. The labelled compound can also be used as precursor, as it is obtained in a sufficient radioactivity amount and specific activity to allow synthesis of other ^{11}C-labelled compounds. A large number of new tracers can be synthesized simply by adding a second enzymatic reaction to the synthesis described above. As an application of the potential use of [^{11}C]acetyl coenzyme A, the synthesis of [^{11}C]N-acetyl-D-glucosamine by means of the enzyme glucosamine acetyltransferase (EC 2.3.1.3) will be worked out.

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