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ENZYMATIC SYNTHESIS OF CARBON-11 *N*-ACETYL-D-GLUCOSAMINE

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SUMMARY

An enzymatic synthesis of [^{11}C] *N*-acetyl-D-glucosamine is described. $^{11}CO_2$ is reacted with methylmagnesium bromide to form [1-^{11}C]acetate. The latter is converted to [^{11}C]acetylcoenzyme A by passage over an enzyme reactor containing immobilized acetylcoenzyme A synthetase (Acetate : CoA ligase (AMP forming) ; EC 6.2.1.1).

[^{11}C]Acetylcoenzyme A is purified on-line and concentrated on an anionexchange column. Elution is achieved with 1.5 M glucosamine HCl pH 8.0 and the [^{11}C]acetylcoenzyme A fraction is collected in an ultrafiltration cell containing the enzyme glucosamine *N*-acetyltransferase (acetylcoenzyme A: 2-amino-2-deoxy-D-glucose *N*-acetyltransferase EC 2.3.1.3).

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After reaction the mixture is ultrafiltrated and the produced [^{11}C] *N*-acetyl-D-glucosamine is purified. Traces of unreacted [^{11}C]acetylCoA and excess of D-glucosamine are removed by passage over an anion- and cationexchange resin respectively.

25 mCi with a specific activity of 125 mCi/μmol are produced in 5-6 ml solution suitable for intravenous injection. The whole synthesis starting from EOB takes about 1 h.

Key Words : Carbon-11 *N*-acetyl-D-glucosamine, enzymatic synthesis, glycoconjugate precursor.

INTRODUCTION

GlcNAc (*N*-acetyl-D-glucosamine ; 2-acetamido-2-deoxy-D-glucose) is a precursor of the biochemical glycoconjugate synthesis. Experiments performed with intravenously and subcutaneously injected ^{3}H-labelled GlcNAc in mice and rats indicate a large uptake in the liver The labelled compound also passes the blood brain barrier and is equally distributed in the different brain areas (1,2). It can be expected that most metabolic active tissues such as tumor tissues will also accumulate this compound.

In order to confirm and extend these results with in vivo experiments, the synthesis of carbon-11 labelled *N*-acetylglucosamine was elaborated. Carbon-11 is a positron emitter with a half-life of 20.4 min. [^{11}C]*N*-acetyl-D-glucosamine is labelled in the carbonyl function of the acetyl group using a specific enzyme catalyzed reaction (Fig. 1).

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

$$^{11}CO_2 + CH_3MgBr \longrightarrow CH_3{}^{11}COOH$$

$$CH_3{}^{11}COOH + ATP + CoASH \xrightarrow[Mg^{2+}\ (EC\ 6.2.1.1)]{\text{Immobilized acetylcoenzyme A synthetase}} CoAS{}^{11}COCH_3 + AMP + PPi$$

$$CoA\text{-}S\text{-}{}^{11}COCH_3 + D\text{-glucosamine} \xrightarrow[(EC\ 2.3.1.3)]{\text{glucosamine } N\text{-acetyltransferase}} N\text{-}CH_3{}^{11}CO\text{-}D\text{-glucosamine} + CoA\text{-}SH$$

Fig. 1. Scheme for the synthesis of [^{11}C]*N*-acetyl-D-glucosamine.

EXPERIMENTAL

Materials

Acetylcoenzyme A synthetase (ACS) from baker's yeast, trilithium coenzyme A (CoA), disodium adenosine-5'-triphosphate (ATP), trilithium acetylcoenzyme A were purchased from Sigma Chemical Company (USA). Glucosamine HCl and other products were obtained from Janssen Chimica (Belgium). p-Chloromercuribenzoate immobilized on agarose beads was obtained from Pierce and DEAE silica (20-40 µm particle size, 100 Å pore size) from Serva Feinbiochemica GmbH. The ionexchange resins AG 1-X8 and AG 50W-X8 were from Bio-Rad Laboratories. The ultrafiltration cell was a Model 3 stirred cell from Amicon provided with a Diaflo PM 10 membrane. The enzyme glucosamine *N*-acetyltransferase was purified from chicken liver.

ACS reactor preparation

The enzyme assay and the immobilization on CNBr

activated controlled pore glass beads (CPG 500, 125-180 µm) were carried out as described elsewhere. However the enzyme/carrier ratio was increased. Five instead of 2.4 U ACS were added to 100 mg derivatized carrier resulting in a specific enzyme activity of 12.0 U/g instead of 6.1 U/g carrier (3,4). The reactor (13.0 x 0.5 cm) contains 1 g of enzyme-loaded glass beads.

Glucosamine *N*-acetyltransferase assay and unit definition

The enzyme glucosamine *N*-acetyltransferase (acetylcoenzyme A : 2-amino-2-deoxy-D-glucose *N*-acetyltransferase EC 2.3.1.3) is isolated and purified from chicken liver. Details concerning the purification procedure are given elsewhere (5). The enzyme assay is based on the HPLC determination of the produced GlcNAc. Five µl purified enzyme concentrate were incubated for 5 min at 30 °C in a final volume of 150 µl containing 75 µmol D-glucosamine HCl/KOH pH 8.0 and 0.12 µmol acetylcoenzyme A. The reaction was stopped by adding 25 µl 4 M H_3PO_4. After filtration (Acro LC 3A 0.45 µm filters, Gelman), 25 µl were injected into the chromatographic system. The column was an Aminex HPX-87C column (300 x 7.8 mm ; BioRad) provided with a recommended guard column and was eluted at 50 °C with double distilled water using a flow of 0.6 ml/min. UV detection at 193 nm was applied.

Using the peak heights and an external standard curve of GlcNAc, the enzyme activity was calculated.

One unit is defined as that amount of enzyme which catalyses the production of one µmol GlcNAc per minute under the described conditions.

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

All parts of the set-up are connected by teflon tubing (0.8 mm i.d.). Electromagnetic valves are used for remote-controlling of the system. $^{11}CO_2$ is produced by irradiation of N_2 gas (10.5 bar) with 18 MeV protons at 15 µA for 20 min. The irradiated target gas is released to a hot cell where $^{11}CO_2$ is frozen out into a stainless steel coil (A ; 2 m x 2.1 mm i.d.) immersed in liquid argon (flow rate 4 $l.min^{-1}$).

The stainless steel coil with the trapped $^{11}CO_2$ is immersed in a water bath at room temperature. The $^{11}CO_2$ is swept out by a dry argon flow (16 $ml.min^{-1}$) and into a reaction vessel (B ; 70 x 10 mm i.d., plugged with a rubber septum) containing 1.2 ml 0.05 M methylmagnesium bromide (50 µl 1.1 M methylmagnesium bromide diluted to 1.2 ml with dry diethyl ether) at room temperature under a dry argon atmosphere. The methylmagnesium bromide in diethyl ether was prepared according to Slegers et al (6).

After the trapping of $^{11}CO_2$, 300 µl methanol are added to destroy the excess of Grignard reagent. The mixture is evaporated to dryness by rinsing with an argon flow and by carefully immersing the reaction vessel in an oil bath at 200 °C. Four hundred µl 0.4 M hydrochloric acid are added to dissolve the residue. The [1-^{11}C]acetic acid is distilled into a reaction vial (C) containing 500 µl 0.1 M potassium phosphate pH 7.6 and 5 µmol KOH. After collection of the [1-^{11}C]acetic acid, 5 µmol CoASH, 10 µmol ATP and 20 µmol $MgCl_2$ are added to vial C by means of a peristaltic pump (D). By a second peristaltic pump (F) and a sample loop the content of the vial is sent over the acetylcoenzyme A synthetase reactor (E ; 13.0 x 0.5 cm i.d.) at 37 °C. The enzyme column is eluted with 0.1 M potassium phosphate pH

7.4 at 1 ml.min^{-1}. When the ^{11}C activity reaches the p-chloromercuribenzoate gel (G ; 5.6 x 1.25 cm i.d.) the flow is increased to 2.0 ml/min. On-line concentration is achieved using an anionexchange column (H ; DEAE Si 100 ; 3.5 x 1.06 cm i.d.). [^{11}C]Acetylcoenzyme A is retained. Traces of unreacted [1-^{11}C]acetate pass the column to the waste. When the ^{11}C activity has accumulated on the anionexchanger, elution is started with freshly prepared 1.5 M glucosamine HCl/KOH pH 8.0.

The fraction containing [^{11}C]acetylcoenzyme A (1.1 ml) is collected in the Amicon ultrafiltration cell (I). The latter is placed on a magnetic stirrer and is previously filled with 250 µl solution containing 0.2-0.4 units glucosamine *N*-acetyltransferase and 0.675 µmol dithiothreitol. After reaction for 4-6 min at room temperature, the ultrafiltration cell is pressurized to 3.7-4.0 bar with nitrogen or argon gas. Under constant stirring, the ultrafiltrate is sent to an anion- and cationexchanger. Further elution is obtained with a peristaltic pump (J) and 0.1 M sodium phosphate pH 6.0 at 1.0 ml.min^{-1}.

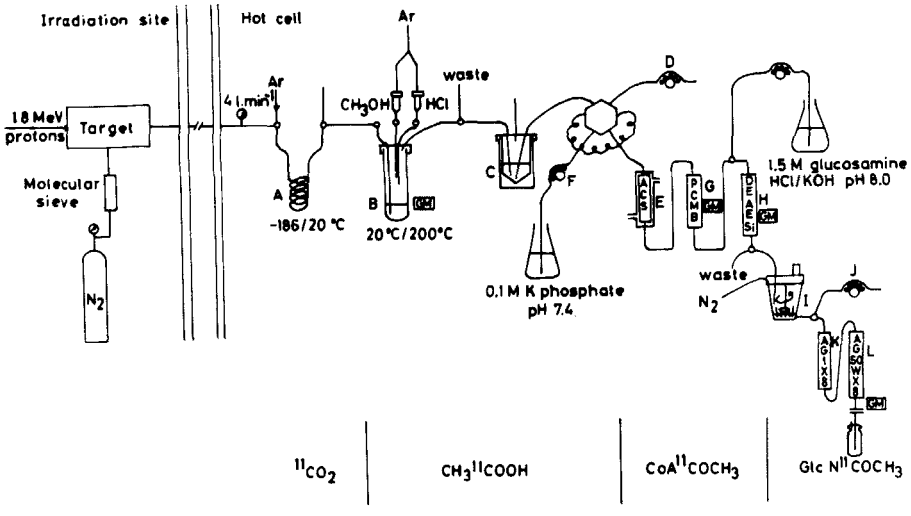

Fig. 2. Set-up for [^{11}C] *N*-acetyl-D-glucosamine synthesis.

The anionexchanger (K ; AG 1-X8 ; 3.9 x 0.8 cm i.d.) retains traces of unreacted [^{11}C] acetylcoenzyme A and the cationexchanger (L ; AG 50W-X8 ; 11 x 1.06 cm i.d.) retains the excess of glucosamine. [^{11}C] GlcNAc is not retarded and is collected in about 5-6 ml with a final pH of 6.6. To monitor radioactivity G.M. tubes are placed at each column.

Conditioning of the columns

Before use, the acetylcoenzyme A synthetase reactor, the p-chloromercuribenzoate gel and the DEAE Si 100 column are equilibrated each with 50 ml 0.1 M potassium phosphate pH 7.4. After the synthesis the enzyme reactor is stored aseptically at 4 °C under 0.5 M potassium phosphate pH 7.4 containing 10 mM glutathione and 0.5 mM EDTA. The p-chloromercuribenzoate gel is washed with 10 bedvolumes (25 ml) water, 10 bedvolumes of a mixture of 10 mM $HgCl_2$ and 20 mM EDTA in 50 mM sodium acetate pH 4.8, followed by 25 bedvolumes of a solution containing 0.2 M NaCl and 1 mM EDTA in 50 mM sodium acetate pH 5.0 (7). The gel is stored at 4 °C under 0.02 % (w/v) NaN_3. The DEAE Si column is washed with 50 ml 1 M HCl followed by 200 ml double distilled water.

The ionexchange resins are preconditioned in 0.1 M sodium phosphate pH 6.0. After use they are washed with 100 ml 1.5 M NaCl and stored under the equilibration buffer.

RESULTS AND DISCUSSION

On-line purification of [^{11}C]acetylcoenzyme A

The [^{11}C] acetylcoenzyme A leaving the acetylcoenzyme A synthetase reactor contains an excess of cofactors of the enzymatic reaction (ATP, CoA, $MgCl_2$) and traces of unreacted [1-^{11}C] acetate. Coenzyme A is a non-competitive inhibitor

of the subsequent glucosamine *N*-acetyltransferase reaction and has to be eliminated. This is achieved by means of an affinity chromatography column containing immobilized p-chloromercuribenzoate. The latter interacts with the thiolfunction of CoASH. With 0.1 M potassium phosphate pH 7.4, [1-^{11}C]acetate elutes first and is partly separated from [^{11}C]acetylCoA. The latter is obtained in a final volume of 17 ml. Coenzyme A is completely retained.

To eliminate [1-^{11}C]acetate and to concentrate [^{11}C]acetylCoA, anionexchange chromatography was used. Separation could easily be obtained using an AG 1-X8 (Cl^- or phosphate) resin (7.0 x 0.3 cm). [1-^{11}C]acetate is not retarded while [^{11}C]acetylCoA is completely retained. However elution of [^{11}C]acetyl CoA with solvents containing high concentrations of highly selective counterions (Cl^-, citrate) always resulted in tailed elution profiles. This effect is due to strong nonspecific adsorption of aromatic compounds on this resin. Moreover, when using the [^{11}C]acetylCoA elution fraction of the AG 1-X8 column, the glucosamine *N*-acetyltransferase reaction was strongly inhibited. Better results were obtained with an anionexchanger based on a silica matrix (DEAE Si). A DEAE Si 100 column (3.45 x 1.06 cm) equilibrated in 0.1 M potassium phosphate pH 7.4 only retains [^{11}C]acetylCoA. By this way [1-^{11}C]acetate is separated. [^{11}C]AcetylCoA can easily be eluted with high Cl^- concentrations (1.5 M). 87 % of the acetylCoA amount elutes in 1.1 ml.

Ultrafiltration cell performance

Immobilization of glucosamine *N*-acetyltransferase onto differently derivatized controlled pore glass beads was unsuccessful. Either a low specific activity (U/g glass) or a bad storage stability was obtained. Therefore the

enzymatic reaction was performed in solution. To remove the enzyme, the mixture was filtered through an ultrafiltration membrane (Diaflo PM 10 ; MW cutoff 10000, Amicon) placed in a commercially available ultrafiltration cell (Amicon).

The performance of the ultrafiltration membrane was checked by measuring the enzyme activity and the protein concentration in the ultrafiltrate. Using the HPLC assay, no enzyme activity could be detected. The protein concentration was below the detection limit (1 µg/ml) of the Bio-Rad protein microassay.

Glucosamine *N*-acetyltransferase reaction

The amount of enzyme needed for a high conversion of acetylcoenzyme A into GlcNAc was tested with 0.8 µmol unlabelled acetylcoenzyme A, which is about the carrier amount present in the cyclotron productions. Acetylcoenzyme A was loaded onto the DEAE Si column and eluted with 1.5 M glucosamine HCl/KOH pH 8.0. The acetylCoA fraction (1.1 ml) was incubated in the presence of varying amounts of glucosamine *N*-acetyltransferase. The enzyme reaction was performed at 30 °C in the presence of 0.5 mM dithiothreitol. GlcNAc production was determined by HPLC at different reaction times as described in the experimental section. Addition of an antioxidans has a positive effect on the reaction. 0.5 mM Dithiothreitol increases the enzymatic acetylation with a factor 1.3. Under these reaction conditions no chemical acetyltransfer from acetylcoenzyme A to dithiothreitol was observed. Using this enzyme reaction, 90 % conversion of acetylcoenzyme A can be obtained (Fig. 3 A).

The time to reach this % conversion varies with the added amount of enzyme.

On the other hand, the radioactivity lost by decay

should also be considered. Therefore the ^{11}C decay profile was superposed on the conversion curves. The two parameters, % conversion and % remaining ^{11}C activity, were multiplied resulting in the overall-yields shown in Fig. 3 B (symbols see Fig. 3 A). The optimal reaction time was 4 min for 0.375 U, 5 min for 0.225 U, 8 min for 0.150 U and more then 10 min for 0.075 U.

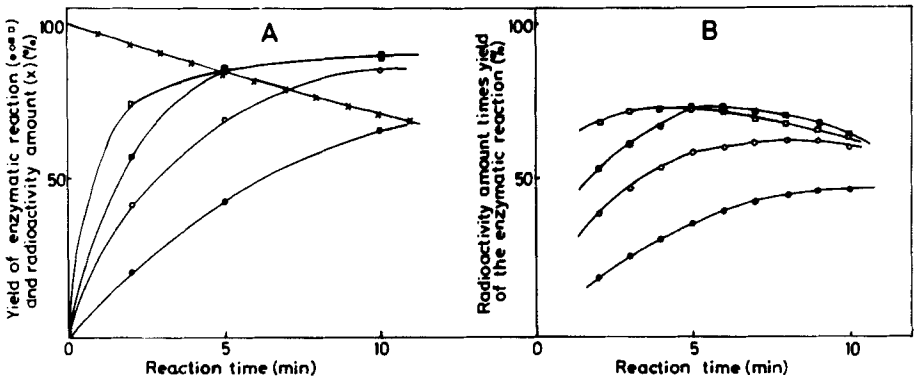

Fig. 3. A. Radioactivity decay (x) and yield of the enzymatic reaction as function of reaction time and added enzyme units : (•) 0.075 U ; (o) 0.150.U ; (■) 0.225 U ; (□) 0.375 U.

B. Yield of radioactivity amount incorporated in [^{11}C] GlcNAc as function of reaction time and enzyme units.

When applying 3.7 bar nitrogen pressure, the ultrafiltration time varied between 10-12 min.

Radiochemical purity and specific activity of [^{11}C]GlcNAc

Radiochromatography of the purified ultrafiltrate was performed using HPLC. An Aminex HPX-87C column (300 x 7.8 mm, Bio-Rad Laboratories) was eluted at 50 °C with double distilled water. Twenty five µl of the final [^{11}C] GlcNAc

preparation were injected and both radioactivity (NaI/Tl) and UV detection at 193 nm were applied. A single radioactive peak was obtained which coeluted with the GlcNAc UV peak at 11.3 min. A typical radiochromatogram is shown in Fig. 4. Since this column completely retains acetylCoA, the presence of the latter was checked using the reversed-phase system previously described (3). No [^{11}C]acetyl CoA could be detected.

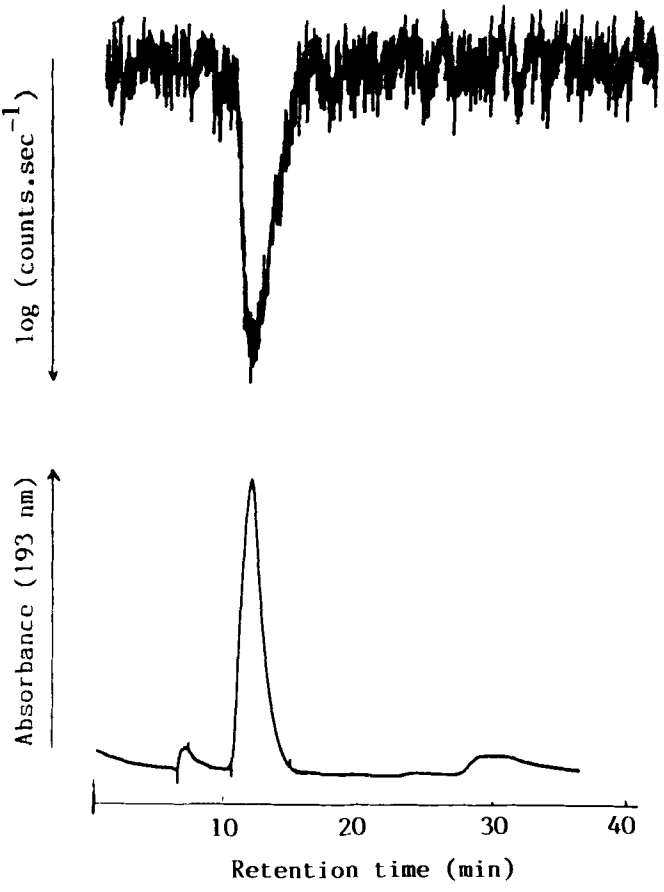

Fig. 4. Radiochromatography of the purified [^{11}C]GlcNAc.

Experiments on low radioactivity level indicate that 25-33 % of the $^{11}CO_2$ radioactivity is incorporated into [^{11}C]GlcNAc (corrected for radioactivity decay). When irradi-

ating with 18MeV protons at 15 μA for 20 min, 25 mCi were obtained with a specific activity of 125 mCi/μmol.

The whole synthesis starting from the end of bombardment takes about 1 h.

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