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Research Article

Chemical and enzymatic synthesis of tritium labelled coenzymes

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Summary

Details of the synthesis of tritium labelled co enzymes-nicotinamide adenine dinucleotide and coenzyme A by isotope exchange and enzymatic reactions are reported. It was established, that among the investigated chemical reactions, the most effective is solid state isotope exchange with gaseous tritium. This method was used to produce [^{3}H] nicotinamide adenine dinucleotide (111 Ci/mmol), [^{3}H] coenzyme A (3.9 Ci/mmol) and D-[G-^{3}H] pantothenic acid (43 Ci/mmol). It was shown that most of the tritium in the labeled nicotinamide adenine dinucleotide and coenzyme A was localized in the nicotinamide (98%) and adenine (89%) sites, respectively. For synthesis of coenzymes labelled with tritium at other sites we developed enzymatic methods which used labelled precursors. Optimum conditions for enzymatic synthesis of [adenine-^{3}H] nicotinamide adenine dinucleotide from [2,8-^{3}H] ATP and [pantothenate-^{3}H] coenzyme A from D-[G-^{3}H] pantothenic acid were determined. The tritium labelled acetyl coenzyme A was synthesized by acetylation of labelled coenzyme A with acetic anhydride. The methods chosen allow one to produce tritium labelled coenzymes at high specific activity. Copyright © 2003 John Wiley & Sons, Ltd.

Key Words: tritium; hydrogen isotope exchange; nicotinamide adenine dinucleotide; coenzyme A; pantothenic acid; enzymatic synthesis

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Introduction

Nicotinamide adenine dinucleotide (NAD, Figure 1a) and coenzyme A (CoA, Figure 1b) are universal molecules in the living world. They play an important role in the metabolism of low-molecular compounds and in energy accumulation processes. NAD^+ is the major acceptor of electrons at the oxidation of 'fuel molecules', such as glucose and fatty acids. Another important function of NAD is its involvement in reactions of mono- and poly(ADP-ribosyl)ation, which ensure the regulation of various cellular processes. CoA plays a key role in the processes of oxidation and synthesis of fatty acids.

For studying processes which involve the participation of coenzymes the labelled analogues are sometimes necessary. The objective of this work was to develop chemical and enzymatic methods of selective tritium labelling of NAD and CoA. For this purpose we studied hydrogen isotope exchange reactions of coenzymes with tritium gas both in solution and the solid phase. We also develop enzymatic methods of synthesis with the use of labelled precursors. [2,8-^{3}H] ATP and D-[G-^{3}H] pantothenic acid were chosen for the synthesis of NAD and CoA, respectively. The tritium labelled acetyl coenzyme A was synthesized by acetylation of labelled coenzyme A with acetic anhydride.

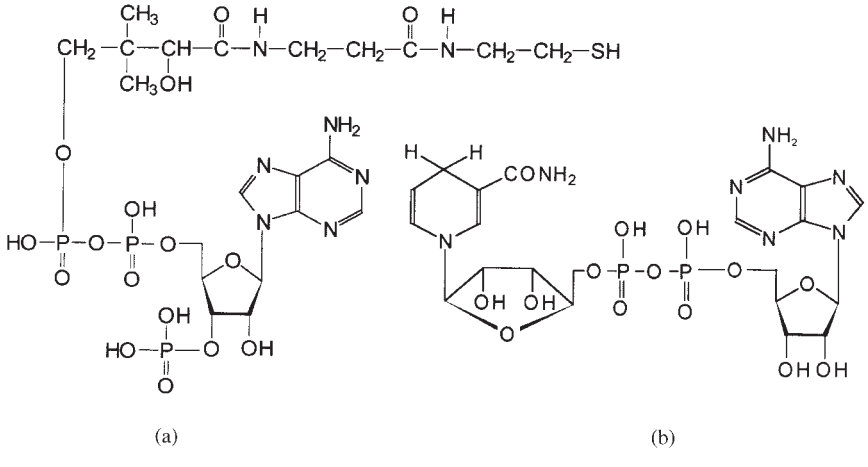

Figure 1. Structures of nicotinamide adenine dinucleotide (a) and coenzyme A (b)

Results and discussion

In the hydrogen isotope exchange between NAD and tritiated water (Table 1, method a) or gaseous tritium (methods b and c) the highest specific activity (A_{sp}) was observed for the solid state isotope exchange reaction with gaseous tritium (method c). High values of A_{sp} and yield of NAD were obtained both for hydrogen isotope exchange in solution (method b) and solid state exchange (method c) with tritiated water (method a). The distribution of tritium in the NAD molecule was ascertained by acid hydrolysis. It was established by methods b and c that only 2.2% and 1.3% of the radioactivity respectively was in the adenine part of the NAD molecule, the majority being in the nicotinamide part of the molecule.

We also studied the reaction of solid state catalytic isotope exchange of coenzyme A and D-pantothenic acid (PA) with tritium (method c, Tables 2 and 3). Table 2 shows the effect of different catalysts and temperature on the yield and specified radioactivity of the CoA produced by this reaction. Catalyst A is the first choice for the synthesis of labelled CoA. Approximately 90% of the tritium in labelled CoA was located in the adenine part of the molecule. The isotope exchange reaction with PA was most efficient with catalyst C (Table 3). The reaction of the barium salt of PA with carries-free tritium (catalyst C) at temperature 180°C resulted in the formation of D-[G-^{3}H] PA with A_{sp} of 43 Ci/mmol and yield of 7.4%.

Table 1. Synthesis of tritium labelled NAD using different methods, Catalyst B

	Methods of synthesis	Specific activity (Ci/mmol)	Yield (%)
a.	Isotope exchange with tritiated water	4.5	17.0
b.	Isotope exchange with gaseous tritium in solution	48.3	17.8
c.	Solid state isotope exchange with gaseous tritium (110°C, 30 min)	111	12.6

Table 2. Solid state catalytic isotope exchange of coenzyme A using gaseous tritium. Incubation time 30 min

Catalyst	T(°C)	Specific activity (Ci/mmol)	Yield (%)
A	140	0.90	58
A	170	2.0	43
A	180	3.9	28
B	190	1.8	37

Table 3. Solid state catalytic isotope exchange of pantothenic acid using gaseous tritium-protium mixture (1:1000 v/v). Incubation time 30 min.

Catalyst	T(°C)	Characteristics of tritium labelled PA	
		A_{sp} (Ci/mol)	Yield (%)
A	170	8.2	32
C	170	25	30
C	180	52	11
C	190	120	2.4

Selectively labelled adenine and pantothenate sites in NAD and CoA are required for investigation of protein transferase reactions and for other purposes. To produce these compounds, the one stage enzymatic synthesis was studied. The enzymatic methods described earlier[1–3] were designed for the synthesis of higher quantities of coenzymes and gave low yields of the final products.

The enzymatic synthesis of tritium labelled NAD was carried out with [2,8-^{3}H] ATP, nicotinamide monoculeotide (NMN), and enzyme nicotinamide nucleotide adenylytransferase (NNAT) from baker's yeast (*Saccharomyces cerevisiae*):

$$[2,8\text{-}^3\mathrm{H}]\mathrm{ATP} + \mathrm{NMN} \leftrightarrow [\text{adenine-}2,8\text{-}^3\mathrm{H}]\mathrm{NAD} + \mathrm{PP_i}$$

The reversibility of the reaction, enzyme inactivation and presence of phosphodiesterase activity in the enzyme preparation are the main limitation of this process. The high dependence of the NAD yield on the initial concentrations of NMN, ATP, and enzyme were determined (Table 4). We determined the optimum reaction conditions to be as follows: NMN – 5 mM, ATP – 1 mM; enzyme – 3 mg/ml; incubation time – 1 h. Under these conditions the yield, as based on a radioactivity, was 85–90%.

For the synthesis of tritium labelled CoA we utilized permeable cells from bacteria *Brevibacterium ammoniagenes* that catalysed complete transformation of D-pantpthenic acid to CoA.

$$\mathrm{PA} \rightarrow 4'\text{-phospho-PA} \rightarrow 4'\text{-phosphopanthenoyl-L-cysteine} \rightarrow 4'\text{-phophopantetheine} \rightarrow \text{dephospho-CoA} \rightarrow \mathrm{CoA}$$

The total equation for the reaction with [G-^{3}H] PA as initial compound looks as follows:

$$[\text{G-}^3\mathrm{H}]\mathrm{PA} + \text{L-cysteine} + 4 * \mathrm{ATP} \rightarrow [\text{pantothenate-}^3\mathrm{H}]\mathrm{CoA} + \mathrm{CO_2} + 3{*}\mathrm{ADP} + \mathrm{P_i} + \mathrm{PP_i}$$

Table 4. The effect of reagent concentration on the yield of tritium labelled NAD in the enzymatic synthesis. Incubation time 1 h at 37°C

Concentration of reagent			Yield (%)
ATP (mM)	NMN (mM)	NNAT (mg/ml)	
0.1	0.5	0.3	28.9
		1.0	49.5
0.3	1.5	1.0	55.3
		3.0	55.9
0.6	3.0	2.0	71.3
		6.0	72.2
1.0	5.0	3.0	85.2
		10.0	79.5

Table 5. The effect of reagent concentration on the yield of tritium labelled CoA in the enzymatic synthesis

Concentration of reagent		Total product yield (%)	Yield of labelled CoA (%)
Enzyme preparation (mg/ml)[a]	5	68.7	ND
	20	90.7	0.6
	100	91.0	23.8
Pantothenic acid (mM)[b]	1	94.8	11.0
	2	84.0	23.5
	4	76.9	28.5
	7	73.7	3.2
	10	34.4	ND

Incubation 15 h at 37°C.
[a] PA – 1.4 mM; Cys – 5 mM; ATP – 10mM.
[b] *B. Ammoniagenes* – 100 mg/ml; the molar ratio of PA, Cys and ATP – 1:2:6.

The final yield depended in a complicated manner on the concentrations of permeable cells and PA (Table 5). We identified the optimum concentrations of reagents to be as follows: PA – 4 mM; ATP – 30 mM; L-cysteine – 15 mM; *B. ammoniagenes* – 100 mg/ml. Under these conditions the yield of CoA reached 35%.

The acetylation reaction of [pantothenate-^{3}H] CoA (see Experimental) allowed us to produce acetyl-[pantothenate-^{3}H] CoA with a high yield (92.8%) and A_{sp} of 39.6 Ci/mmol.

Experimental

The catalysts used were: 5% Pd/$BaSO_4$ (A) and 5% Pd/$CaCO_3$ (B) (Fluka), 5% Pd/$CaCO_3$ (C) (Aldrich) and PdO (Merck).

Table 6. HPLC of tritium labelled compounds. Nucleosil 120-5, C18, 4.6 × 250 mm

Compound	Mobile phase	Retention time (min)
NAD	2%CH_3CN in 0.1 M TEAB (pH 7.0), 0.5 ml/min	10.2
CoA	5% CH_3CN in 220 mM NaH_2PO_4 (pH 5.3) and 0.45 M dithiothreitol (DTT), 1.0 ml/min	24.0
Acetyl-CoA	10% CH_3CN in 0.1 M TEAB (pH 6.5), 1.0 ml/min	14.3
Pantothenic acid	2% CH_3CN in 0.1 M TEAB (pH 6.2), 1.0 ml/min	16.4

All the labelling procedures were carried out in an installation suitable for manipulations with gaseous tritium.[4]

TLC was performed on PEI-cellulose plates (Merck), solvents – 1 mol/l NaCl or distilled water and on Silufol silicagel plates in butanol–acetic acid–amonia–acetone–water (35:15:3:25:15 v/v/v/v).

We carried out the purification of [^{3}H] NAD in two stages: (1) by chromatography on DEAE-cellulose column (16 × 250 mm), elution (60 ml/h) by linear gradient 0.05–0.15 M (8 h) of triethylammonium bicarbonate (TEAB) pH 7.5; and (2) by HPLC (see Table 6). The acetyl-[^{3}H] CoA was also purified by this procedure (Table 6).

UV absorption spectra were measured using a Specord M-40 spectrophotometer (Germany). Sample radioactivity was measured on a β-radioactivity counter using LC-8 as scintillation liquid (NPO 'Monocrystallreactiv', Russia).

Isotope exchange reaction with high-specific activity tritiated water: The tritiated water was synthesized by a method described previously.[5] The tritiated water was placed in a reaction vial containing 10 mg of catalyst and 1.5 mg of NAD. The mixture was stirred at room temperature for 16 h. After the incubation, the catalyst was separated by centrifugation and the solvent removed in vacuum. Labile tritium was removed by dissolving residue in 10 ml of 50% ethanol and further evaporation. This operation was repeated twice.

Isotope exchange with gaseous tritium in solution: 2.2 mg of NAD in 100 µl of water and 10 mg of catalyst A were placed in a glass reaction vial. The vial was connected to a tritium gas line. The vial was then frozen in liquid nitrogen, evacuated and filled with gaseous tritium up to the pressure of 300 mm Hg. After defrosting, the reaction mixture was stirred at room temperature. After removal of gas, the catalyst was

separated by filtration. Labile tritium was removed by evaporation at 35–37°C. The dry residue was dissolved in water, and the labelled compound isolated.

Solid state catalytic hydrogenation (SCH): A solid mixture of the initial compound and the catalyst was place in a glass reaction vial that was then connected to a system for handling gaseous tritium, evacuated and tritium gas introduced into the vial at 300 mm Hg pressure. The vial was thermostated at the chosen temperature, then cooled and the excess tritium removed from the vial. The reaction products were washed with water, and the catalyst was separated by filtration. Labile tritium was removed by evaporation of the filtrate to dryness. The end product was isolated by chromatography as described above.

Analysis of intramolecular distribution of tritium: The coenzymes were hydrolysed under acid conditions. 0.25 Mg of coenzyme A (0.3 μmol) was dissolved in 0.3 ml of NaH_2PO_4 (pH 4.4), placed in a sealed vial and kept at 100°C for 45 h. 0.20 mg of NAD (0.3 μmol) was dissolved in 0.3 ml of 0.01 M HCl placed in the sealed vial and kept at 100°C for 30 min. After hydrolysis the AMP was isolated and its A_{sp} was determined.

Enzymatic synthesis of tritium labelled NAD: The enzymatic preparation of nicotiamide nucleotide-adenyliltransferase from *S. cerevisiae* was prepared as described early.[6] The synthesis of [adenine-^{3}H] NAD was carried out in the following way: 39 mCi of [2,8-^{3}H] ATP (34 Ci/mmol) was placed in a 50 ml flask, the solvent removed by evaporation and the mixture (0.8 ml) of 0.1 M tris-HCl (pH 7.5) (500 μl), 1 M $MgCl_2$ (150 μl), 50 mM NMN (100 μl) and deionized water (200 μl) added. The mixture was pre-incubated for 5 min at 37°C and 5 mg of enzyme in 200 μl of 25 mM tris-HCl (pH 7.4) containing reduced glutatione (2 mM) and ethylenediaminetetraacetic acid (1 mM) was added. After 1 h incubation at 37°C the vial was placed on a boiling water bath for 3 min and then cooled to room temperature. Denaturated proteins and other insoluble components were removed by centrifugation. NAD was purified by DEAE-cellulose and HPLC as described above. Fractions with labelled NAD were combined and TEAB was removed by evaporation. In this way 13.5 mCi of [adenine-^{3}H] NAD with A_{sp} 13.6 Ci/mmol and radiochemical purity 98% was obtained. The NAD was stored as a solution of 1 mCi/ml in 50% ethanol at –20°C.

Enzymatic synthesis of tritium labelled CoA: For the synthesis of [pantothenate-^{3}H] CoA from [G-^{3}H] PA we utilized permeabilized cells

of *B. ammoniagenes* as described in the literaure.[3] The reaction mixture contained the following reagents: 86 mCi (2 μmol) of D-[G-^{3}H] pantothenic acid; 750 μl of 0.5 M potassium-phosphate buffer (pH 6.5); 30 μl of 0.5 M $MgSO_4$, 150 μl of 50 mM L-cysteine (30 μl), 150 μl of 100 mM ATP, and 420 μl of H_2O. The mixture was carefully stirred and pre-incubated for 10 min at 37°C. Then 150 mg of permeable cells were added and the mixture incubated for 16 h at 37°C. After incubation the mixture centrifuged for 20 min at 6000 rpm.The pellet was washed with 3 ml of water and centrifuged as described above. The supernatants were combined and diluted with H_2O to 5 ml. Then 2-mercaptoethanol was added and the mixture kept at 30°C for 1 h. The labelled CoA was purified by HPCL. As a result we obtained 25.5 mCi of CoA with A_{sp} of 40 Ci/mmol and purity 98%. The CoA prepared was stored as a solution (1 mCi/ml) in 50% ethanol at –20°C.

Acetylation of [pantothenate-^{3}H]coenzyme A: For the preparation of microquantity of labelled acetyl-CoA we modified the procedure described in Ref.[7] To 14 mCi of coenzyme A (0.35 μmol) in 44 μl of deionized water was added 4 μl of 1 M $KHCO_3$ and the mixture cooled to 0°C. Then 4.6 μl of freshly prepared 0.1 M acetic anhydried in water (0°C) was added and the mixture kept for 5 min at 0°C. then the solution was neutralized with 0.1 M TEAB and evaporated under vacuum. TEAB was removed by twice dilution/evaporation procedure with 50% ethanol as solution. The dry residue was dissolved in 0.5–1.0 ml of water and fractionated on a DEAE-cellulose column. Fractions with acetyl-[^{3}H] CoA were combined and evaporated under vacuum. TEAB was removed as previously described. The final purification was performed by HPCL. After purification we obtained 13 mCi of acetyl-[^{3}H] CoA with A_{sp} of 39 Ci/mmol.

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