NON-ENZYMIC HYDROGEN EXCHANGE BETWEEN NICOTINAMIDE ADENINE DINUCLEOTIDES*

Julio Ludowieg and Albert Levy

Department of Orthopaedic Surgery and the Rheumatic Disease Group, Department of Medicine, University of California, San Francisco Medical Center

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In the course of studying the mechanism of reduction of NAD^+ by 1-n-propyl-1,4-dihydronicotinamide (PrNDH) (Ludowieg and Levy, 1962), an isotopic exchange was observed between the oxidized and reduced partners involved in this reaction.

The stereospecificity and hydrogen exchange between dinucleotides was studied with the aid of tritium. The amount of tritium in the reduced nucleotides was determined after separation of the reaction products by DEAE-cellulose (Pastore and Freidkin, 1961). Nicotinamide-4-t dinucleotides, $NAD(T)^+$ and $NADP(T)^+$ were prepared by the cyanide exchange method (San Pietro, 1955) and purified by charcoal (LePage and Mueller, 1949) (Ludowieg and Vennesland, unpublished). The nicotinamide was obtained by alkaline hydrolysis of the oxidized dinucleotide (Marcus, et al., 1953), followed by ether extraction and crystallization in benzene (Loewus, et al., 1963). The analogs, 1-n-propyl nicotinamide-4-t iodide ($PrND(T)^+$-iodide) and PrNDH were prepared according to Karrer, et al., (1937). In the exchange between PrNDH and $PrND(T)^+$-iodide, the reduced

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compound (PrNDH) was extracted at the end of the reaction with $CHCl_3$ and the solvent evaporated in vacuo to dryness. The residue was dissolved in H_2O and the dihydro derivative was diluted by heating the solution with non-isotopic PrNDH. Pure PrNDH crystallizes out upon cooling and seeding.

An outline for the isotopic exchange between $NAD(T)^+$ and NADH is represented in equations (a) and (b). The enzymic reoxidation of NADH(T) with acetaldehyde is stereospecific with respect to the reduced position of the dihydronicotinamide ring (Fisher, et al., 1953). Therefore, the isolation of ethanol by lyophilization of the medium and the nicotinamide from the remaining reoxidized $NAD(T)^+$, (equation b), determine the specific activity as well as the isotopic distribution in the reduced dinucleotide.

(a) $NAD(T)^+ + NADH \rightleftharpoons NADH(T) + NAD^+$

(b) $NADH(T) + \text{acetaldehyde} \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} NAD(T)^+ + \text{ethanol(T)}$

The results of these experiments are shown in Table I. In Expt. 1, the total radioactivity (ethanol + nicotinamide) present in the reduced dinucleotide is about 10% of what would be expected for a complete exchange between the reduced and oxidized dinucleotides. In Expt. 2, where there is a longer incubation period, the tritium exchange was more extensive. In this experiment, the radioactivity of the separated NADH (12,400 + 15,200) was 58% of the $NAD(T)^+$ (47,600) used as the starting radioactive component in this system. Also, Expts. 1 and 2 show that the tritium is about equally distributed in both sides of the dihydronicotinamide plane.

Table I

Exchange and Tritium Distribution Between Dinucleotides

Expt.	Reaction* Components	Incubation Time	Products Separated	Products Analyzed	c.p.m./ μmole
1.	$NAD(T)^+$	zero		nic.	47,600
	$NAD(T)^+$ + NADH	1 hr.	NAD^+	nic.	42,800
			NADH	nic.	2,040
				ethanol**	2,520
2.	$NAD(T)^+$ + NADH	12 hrs.	NAD^+	nic.	18,600
			NADH	nic.	12,400
				ethanol**	15,200
3.	$NADP(T)^+$	zero		nic.	60,000
	$NADP(T)^+$ + NADP	1 hr.	$NADP^+$	nic.	53,000
			NADPH	nic.	2,800
				H_2O**	3,200
4.	$NADP(T)^+$ + NADPH	12 hrs.	$NADP^+$	nic.	23,200
			NADPH	nic.	16,600
				H_2O**	19,000

*Reaction mixture contained 30 μmoles of each dinucleotide in a final volume of 1 ml. of 0.1M Tris, pH 8.

**Obtained by lyophilization of the medium and control corrected.

The general scheme for the isotopic exchange between $NADP(T)^+$ and NADPH is shown in equations (c) to (e). The reduced dinucleotide was oxidized with oxidized glutathione (G-S-S-G) and glutathione reductase, which catalyzes specifically the transfer of hydrogen from the opposite side of the dihydronicotinamide ring used by alcohol dehydrogenase to the disulfide group of G-S-S-G (Stern and Vennesland, 1960). Any tritium transferred from NADPH(T) to the substrate is present in the H_2O of the medium since the hydrogen of reduced glutathione exchanges freely with H_2O. The specific activity and the isotopic distribution in the reduced dinucleotide is therefore determined from the nicotinamide of $NADP(T)^+$ (equation d) and the H_2O of lyophilization (equation e).

(c) $NADP(T)^+ + NADPH \rightleftharpoons NADPH(T) + NADP^+$

(d) $NADPH(T) + G\text{-}S\text{-}S\text{-}G \xrightarrow[\text{reductase}]{\text{glutathione}} NADP(T)^+ + 2\ G\text{-}SH(T)$

(e) $G\text{-}SH(T) + H_2O \longrightarrow G\text{-}SH + H_2O(T)$

The isotopic exchange between $NADP(T)^+$ and NADPH is shown in Expts. 3 and 4 (Table I). The degree of exchange in this system is about identical with the degree of exchange obtained with the NAD^+-NADH system. The following evidence implicates position 4 as the sole place for the exchange process. Chemical degradation of the nicotinamide by the method of Pullman, et al., (1954) shows no tritium at carbon 2 or 6. Reoxidation of NADH with ketoglutarate and glutamic acid dehydrogenase (opposite stereospecificity to alcohol dehydrogenase (Nakamoto and Vennesland, 1960) rules out any tritium at position 5, since the products of this reaction (glutamate and nicotinamide) were found about equally radioactive. The isotopic exchange between $PrND(T)^+$-iodide and PrNDH is shown in Table II.

Table II

Isotopic Exchange Between PrNDH and $PrND(T)^+$-Iodide

Reaction* Components	Product Isolated	Incubation Time (hrs.)	c.p.m./μmole
$PrND(T)^+$-iodide		zero	28,000
$PrND(T)^+$-iodide	H_2O**	16	87
$PrND(T)^+$-iodide + PrNDH	PrNDH	6	19,500
$PrND(T)^+$-iodide + PrNDH	PrNDH	16	14,500
$PrND(T)^+$-iodide + PrNDH	H_2O**	16	100

*Reaction mixture contained 48 μmoles of $PrND(T)^+$-iodide and 48 μmoles of PrNDH in a final volume of 1 ml. of 0.1 M Tris, pH 10. Incubation temp. 30°.
**H_2O of lyophilization.

To study the nature of the isotopic transfer, composition mixtures similar to the one described in Table I were incubated for 12 hrs. and directly lyophilized. The H_2O of lyophilization contained about 0.6% of the activity of the reaction mixture and the same as control experiments containing the radioactive substrate alone. Thus, the isotopic exchange processes between the reduced and oxidized forms of nicotinamide adenine dinucleotides proceeded directly between the two reacting species. Similar conclusions are drawn for the PrNDH-PrND(T)-iodide exchange, as shown in Table II.

Apparently a direct and non-stereospecific hydrogen transfer without a net oxidation or reduction is a general property of the nicotinamide adenine dinucleotides and analog systems.

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