the observations reported herein: (1) Keister, D. L. (1963), *J. Biol. Chem. 238*, PC2590. (2) Shen, G. M., Yang, S. Y., Shen, Y. K., and Yin, H. C. (1963), *Sci. Sinica Peking 12*, 685.

References

Allen, M. B., and Murchio, J. C. (1963), *Biochem. Biophys. Res. Commun. 11*, 115.

Amesz, J., and Duysens, L. N. M. (1962), *Biochim. Biophys. Acta 64*, 261.

Arnon, D. I. (1949), *Plant Physiol. 24*, 1.

Avron, M. (1960), *Biochim. Biophys. Acta 40*, 257.

Avron, M. (1961), *Biochem. J. 78*, 735.

Avron, M., and Jagendorf, A. T. (1959), *J. Biol. Chem. 234*, 1315.

Bishop, P. M., and Whittingham, C. P. (1963), *Nature 197*, 1225.

Bose, S. K., and Gest, H. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 337.

Geller, D. M., and Lipmann, F. (1960), *J. Biol. Chem. 235*, 2478.

Govindjee, R., Govindjee, M., and Hoch, G. (1962), *Biochim. Biophys. Res. Commun. 9*, 222.

Gromet-Elhanan, Z., and Avron, M. (1963), *Biochem. Biophys. Res. Commun. 10*, 215.

Jagendorf, A. T. (1956), *Arch. Biochem. Biophys. 62*, 141.

Krogmann, D. W. (1958), *Biochim. Biophys. Acta 30*, 655.

Krogmann, D. W. (1960), *J. Biol. Chem. 235*, 3630.

Krogmann, D. W., and Vennesland, B. (1959), *J. Biol. Chem. 234*, 2205.

Levine, R. P., and Smillie, R. M. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 417.

Levine, R. P., and Volkmann, D. (1961), *Biochem. Biophys. Res. Commun. 6*, 264.

Losada, M., Whatley, F. R., and Arnon, D. I. (1961), *Nature 190*, 606.

Mayne, B. C., and Brown, A. H. (1963) *in* Studies on Microalgae and Photosynthetic Bacteria, Japanese Society of Plant Physiologists, eds., Tokyo, University of Tokyo Press, p. 347.

Minakami, S., Ringler, R. L., and Singer, T. P. (1962), *J. Biol. Chem. 237*, 569.

Newton, J. W., and Kamen, M. D. (1957), *Biochim. Biophys. Acta 25*, 462.

Punnett, T. (1959), *Plant Physiol. 34*, 283.

San Pietro, A., and Lang, H. M. (1958), *J. Biol. Chem. 231*, 211.

Trebst, A. V., and Eck, H. (1961), *Z. Naturforsch. 16b*, 455.

Vernon, L. P. (1962), Biologistics for Space Systems Symposium, AMRL-TDR-62-116, 6570th Aerospace Medical Research Laboratories, Wright-Patterson AFB, Ohio, p. 131.

Vernon, L. P., and Ash, O. K. (1960), *J. Biol. Chem. 235*, 2721.

Wadkins, C. L., and Lehninger, A. L. (1959), *J. Biol. Chem. 234*, 681.

Studies on the Nonenzymic Hydrogen Exchange Between Nicotinamide Adenine Dinucleotides*

Julio Ludowieg and Albert Levy

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

Received September 17, 1963

The components of the couple NAD^+-NADH interact with each other. Similar interactions were demonstrated with the components of $NADP^+$-NADPH and (1-*n*-propyl nicotinamide chloride)-(1-*n*-propyl-1,4-dihydronicotinamide) systems. In each case, there is a nonstereospecific hydrogen exchange with no net oxidation-reduction and the formation of a reversible complex. Another colored complex is formed also, but there is no evidence that the formations of these two complexes are dependent upon each other.

Of the numerous enzyme-catalyzed reactions involving NAD^+ and $NADP^+$, the transhydrogenase systems are of biological importance (Colowick *et al.*, 1952; Kaplan, 1961). In these reactions, a direct and stereospecific hydrogen exchange is observed (San Pietro *et al.*, 1955). Among the nonenzymic reactions, the reduction of 1-benzyl-3-acetylpyridinium chloride by NADH and NADPH was described as a model system for the transhydrogenases (Cilento, 1960). A direct and nonstereospecific hydrogen transfer was demonstrated in the nonenzymic reduction of the 3-acetylpyridine analog of NAD^+ by NADH (Spiegel and Drysdale, 1960; Drysdale *et al.*, 1961). The observed oxidation-reduction was attributed to the reactivity of the 1-substituted pyridinium ring for hydride abstraction. Similar systems, wherein a net difference between the E_0 values of the couple dinucleotide systems was observed, were suspected to exchange hydrogen with a net oxidation-reduction. Thus a mixture of NADH and 2-aldehydepyridine adenine dinucleotide gave similar results to those found with the 3-acetylpyridine adenine dinucleotide and 1-benzyl-1,4-dihydronicotinamide reduced 1-methyl-3-acetyl pyridinium iodide (Spiegel and Drysdale, 1960). Recently, PrNDH[1] was shown to rapidly reduce NAD^+ to NADH (Ludowieg and Levy, 1962). While

* Supported by research grants from the Committee on Research, University of California School of Medicine, the Arthritis and Rheumatism Foundation (Northern California Chapter), and the United States Public Health Service (grants 2A-5124 and A-1359).

[1] Abbreviations: NAD^+(T), NADH(T), oxidized and reduced nicotinamide adenine dinucleotide containing tritium at C-4 of the nicotinamide ring; $NADP^+$(T), NADPH(T), oxidized and reduced nicotinamide adenine dinucleotide phosphate containing tritium at C-4 of the nicotinamide ring; $PrND^+$, 1-*n*-propyl nicotinamide as a cation; $PrND^+$-iodide and $PrND^+$-chloride as halides of $PrND^+$; $PrND^+$(T), 1-*n*-propyl nicotinamide with tritium at C-4 as a cation; $PrND^+$(T)-iodide and $PrND^+$(T)-chloride as halides of $PrND^+$(T); PrNDH, 1-*n*-propyl-1,4-dihydronicotinamide; PrNDH(T), tritium labeled PrNDH at C-4; Tris, tris(hydroxymethyl)aminoethane; DEAE-cellulose, diethylaminoethyl-cellulose; GSSG, GSH, oxidized and reduced glutathione.

studying this reduction, it was found that a direct isotopic exchange was also in progress between NAD^+-NADH and ($PrND^+$-chloride)-PrNDH systems (Ludowieg and Levy, 1963). A preliminary account of the properties of these systems is the purpose of this report.

Experimental

Materials.—Crystalline alcohol dehydrogenase, lactic dehydrogenase, glutathione reductase, glutamic acid dehydrogenase, β-NAD^+, and β-$NADP^+$ were obtained from the Sigma Chemical Co. DEAE-cellulose was obtained from Carl Schleicher and Schnel Co., Keene, N. H.

Methods.—NAD^+(T) was prepared by the cyanide-exchange procedure for introducing deuterium into the 4-position of the nicotinamide ring of NAD^+ (San Pietro, 1955). The radioactive dinucleotide was purified by passing the reaction mixture through a column of Darco, followed by elution with 2% pyridine in 40% ethanol-H_2O (LePage and Mueller, 1949; Krakow *et al.*, 1963). The isotopic dinucleotides were 80% pure upon assay with enzyme and contained about 90% tritium at C-4 and 10% at C-2 of the nicotinamide, as determined by the pyridone method of Pullman *et al.* (1954). Nicotinamide-4-T was obtained by heating NAD^+(T) in an alkaline solution of 0.3 M phosphate (Marcus *et al.*, 1958). Under these conditions, hydrolysis removes any tritium at C-2 of nicotinamide (Kaplan, 1955). The isolation and purification of the nicotinamide was carried out as described by Loewus *et al.*, (1953). In experiments where the dinucleotide was purified by DEAE-cellulose, the nicotinamide was repurified by sublimation. This step was done routinely since a contaminant from the cellulose exchanger often was present in the isolated nicotinamide.

NADH(αT) was prepared by reduction of NAD^+(T) (400,000 cpm/μmole) with glutamic acid dehydrogenase and L-glutamate. The reduced dinucleotide was separated in a DEAE-cellulose column (Pastore and Friedkin, 1961). An aliquot of the eluate containing pure NADH(αT) was diluted with nonisotopic NADH to make a solution of 30 μmoles/ml. The nicotinamide-4-tritium labeled derivatives, $PrND^+$(T)-iodide and PrNDH(T) were prepared by micromethods based on Karrer's procedures (Karrer *et al.*, 1936; Karrer and Stare, 1937; Karrer *et al.*, 1937).

Preparation of $PrND^+$(T)-Iodide. —Nicotinamide-4-tritium (300 mg) in 5 ml of 1-propanol containing 1 ml of *n*-propyl iodide was heated in a 10-ml volumetric flask with glass stopper for 4 days at 60°. After the addition of 10 ml of ether and cooling, $PrND^+$(T)-iodide was removed from the solution. Recrystallization in ethanol-H_2O gave light-yellow needles. Yield, 500–600 mg, mp 198°.

Preparation of PrNDH(T).—To a glass tube containing 1000 mg anhydrous Na_2CO_3 dissolved in 4 ml H_2O, 600 mg (3 mmoles) of $PrND^+$(T)-chloride was added. This solution was magnetically stirred and placed in an ice bath. The reaction was initiated by the addition of 600 mg $Na_2S_2O_4$, followed by the passing of N_2 gas through the mixture for 5 minutes. After 45 minutes the orange mixture was extracted with six portions of $CHCl_3$, 10 ml each.[2] The $CHCl_3$ layer was removed, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to dryness at 30–40°. The oily residue was dissolved in a minimal amount of boiling H_2O (10–15 ml). Upon cooling the solution at 0° and seeding, pure PrNDH(T) crystallized out. Yield, 150–200 mg, mp 91–93°. In this method, $PrND^+$(T)-chloride can be replaced by $PrND^+$(T)-iodide, provided that the volume of the reaction mixture is tripled. Crystallization of PrNDH in H_2O has been reported by Anderson and Berkelhammer (1958). Contrary to their observation, PrNDH crystallized in H_2O can be kept in the dark at room temperature in a vacuum desiccator for many months. However, care should be taken to keep this compound dry and away from volatile organic solvents that cause its rapid deterioration. $PrND^+$-chloride was obtained from $PrND^+$-iodide and Dowex 2-Cl^- according to the procedure described for the preparation of 1-methyl nicotinamide chloride (Decker *et al.*, 1962), mp 193–194°, E_{max} 4 × 10^3 at 266 mμ in H_2O. For PrNDH, E_{max} 7.3 at 360 mμ in 0.1 M Tris, *p*H 8.5.

The hydrogen exchange and the stereospecificity of the reaction were studied in mixtures containing the oxidized and reduced dinucleotides, with one of these components being radioactive. Controls were run in which a single (radioactive) component was incubated under the same conditions as those used in the reaction mixture. The amount of tritium present in the reduced and oxidized dinucleotides was determined after purification by adsorption of the reaction mixture in a refrigerated DEAE-cellulose column (5 × 2.5 cm) equilibrated with 0.01 M glycylglycine, *p*H 7.5 (Pastore and Friedkin, 1961). The oxidized dinucleotides were eluted with 0.01 M NaCl in glycylglycine buffer, *p*H 7.5. On the NAD^+-NADH exchange, NADH was eluted with 0.1 M NaCl in 0.01 M glycylglycine, *p*H 7.5. On the $NADP^+$-NADPH exchange, NADPH was eluted with 0.2 M NaCl in 0.01 M glycylglycine, *p*H 7.5. Fractions of 2 ml each were collected and only the fractions which showed the absorption ratio 260/340, corresponding to the pure reduced dinucleotide, were pooled and enzymically reoxidized. No attempts were made for the complete recovery of the reaction products from the DEAE-cellulose column. Other fractions which were suspected to be contaminated were discarded.

The tritium distribution and the extent of the isotope in the nicotinamide ring of the isolated dinucleotides were determined as shown in equations (1) and (2). The enzymic reoxidation of NADH(T) with acetaldehyde is stereospecific with respect to the reduced position of the dihydronicotinamide ring (Fisher *et al.*, 1953). Thus, the isolation of ethanol by lyophilization of the medium and the nicotinamide from the remaining reoxidized NAD^+(T) determines the specific activity as well as the isotopic distribution in the reduced and oxidized dinucleotide. A similar method was proposed for the determination of stereospecificity on nicotinamide dinucleotide–linked enzymes (Fernandez *et al.*, 1962).

$$NAD^+(T) + NADH \rightleftharpoons NADH(T) + NAD^+ \quad (1)$$

$$NADH(T) + \text{acetaldehyde} \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} NAD^+(T) + \text{ethanol}(T) \quad (2)$$

The outline for the isotopic exchange between $NADP^+$-(T) and NADPH is shown in equations (3) to (5). The reduced dinucleotide was reoxidized with GSSG 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 bond of GSSG (Stern and Vennesland, 1960). Any tritium transferred from

[2] A high specific activity PrNDH(T) is obtained by replacing H_2O with tritium-labeled H_2O. In this case the extractions were aided by the use of a mechanical test tube shaker (Cyclomixer, Clay Adams, Inc., N. Y.).

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. Thus the specific activity and the isotopic distribution in the reduced dinucleotide are determined from the nicotinamide of NADP$^+$(T) (equation 4) and the H_2O of lyophilization (equation 5).

$$\text{NADP}^+(\text{T}) + \text{NADPH} \rightleftharpoons \text{NADPH(T)} + \text{NADP}^+ \quad (3)$$

$$\text{NADPH(T)} + \text{GSSG} \xrightleftharpoons[\text{reductase}]{\text{glutathione}} \text{NADP}^+(\text{T}) + 2\text{GSH(T)} \quad (4)$$

$$\text{GSH(T)} + \text{H}_2\text{O} \longrightarrow \text{GSH} + \text{H}_2\text{O(T)} \quad (5)$$

For the tritium exchange between PrNDH and PrND$^+$-(T)-chloride the mixture components were separated by paper chromatography (Whatman 3MM) using 70% 1-propanol in 0.1 M Tris, *p*H 10 at 4°, and were detected with the use of mineral light, lamp Model SL-2537 (Ultraviolet Products, Inc.). A bright fluorescent fast-moving spot corresponded to PrNDH and a dark slow-moving spot corresponded to PrND$^+$-chloride.[3] These compounds were eluted with cold 70% 1-propanol buffer solution and further characterized by their absorption spectra and chemical properties.

The interaction between the oxidized and reduced 1-substituted nicotinamide derivatives was studied by spectrophotometry. Concentrated reaction mixtures were scanned from 400 to 600 mμ in cylindrical cells of known light-path lengths. These solutions were further diluted and scanned in cells wherein the path length was proportionally increased to this dilution. Spectral measurements were obtained with a Cary Model 11 recording spectrophotometer. Aqueous buffered solutions were the usual medium for these experiments. However, because of the limited solubility of PrNDH in H_2O, at final concentration for each reactant of 0.1 M or higher, mixtures in 50% ethanol-buffer solutions were used.

For radioactive measurements samples of the tritium-labeled compounds were dissolved in 6 ml of ethanol to which was added 0.6 ml of H_2O followed by 8.4 ml of the scintillation solution made of toluene containing 6 g/100 ml of 2,5-diphenyl oxazole and 0.1 g/100 ml of 1,4-bis-2-(5-phenyloxazolyl)-benzene and 0.1 g/100 ml of 1,4-bis-2-(5-phenyloxazolyl)-benzene, and counted in the Packard Tri-Carb automatic liquid scintillation spectrophotometer. The efficiency of counting was of the order of 4–5%.

[3] In paper chromatography PrND$^+$-chloride moves as a single component. However, when PrND$^+$-iodide was run on paper chromatography, two components closely separated from each other were spotted by the mineral light lamp. Upon elution with H_2O, the fast-moving spot was identical with the absorption spectrum of PrND$^+$-iodide, and the slow-moving component was identical with the absorption spectrum of PrND$^+$-chloride. The absorption spectrum of a water solution of PrND$^+$-iodide shows two absorption bands with max at 266 mμ ($E = 4 \times 10^3$) and 226 mμ ($E = 16.6 \times 10^3$), and a shoulder at 270 mμ. In the case of PrND$^+$-chloride, the absorption band at 226 mμ is absent. Yet in experiments wherein PrND$^+$(T)-iodide was isolated by paper chromatography the specific activity of the two components was identical. Apparently the iodide anion was responsible for the band with max at 226 mμ (Smith *et al.*, 1956; Kosower *et al.*, 1957). The purity of PrNDH isolated by paper chromatography (as measured by absorption spectra) may not be as great as if it were isolated as a crystalline compound or derivative. The impurities which unavoidably were present in paper chromatography elutions could not amount, however, to a quantity which would make the isotopic results unacceptable. Isotopic data obtained with PrNDH isolated as a pure crystalline compound is in good agreement with the data obtained by isolating PrNDH on paper chromatography (Ludowieg and Levy, 1963).

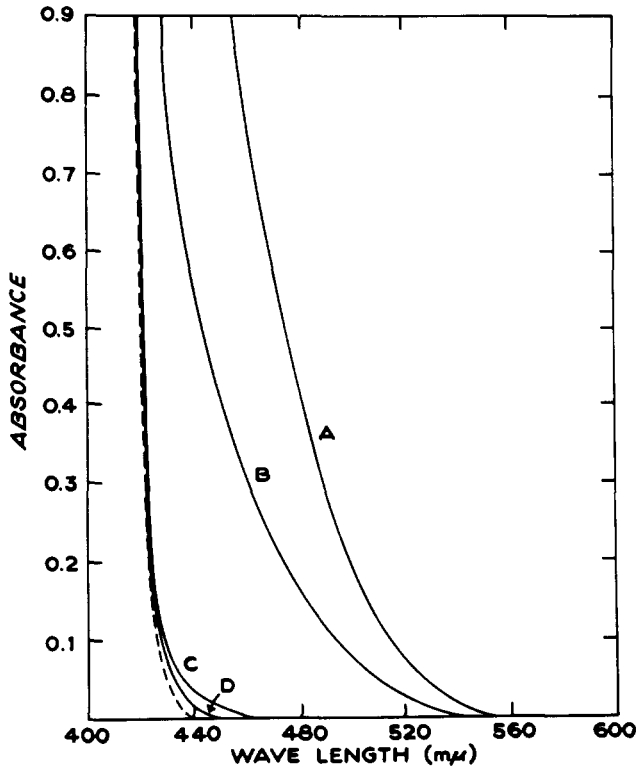

FIG. 1.—Dilution effect on the spectra of PrND$^+$-chloride and PrNDH mixtures. A is 15 mmoles PrNDH and 15 mmoles PrND$^+$-chloride in 10 ml of 50% ethanol in 0.1 M Tris, *p*H 8.5. Spectra were immediately recorded in 0.1-mm cells; B is A after 10-fold dilution, spectra recorded in 1.0-mm cells; C is A diluted 100-fold, spectra recorded in 10.0-mm cells; D is A diluted 1000-fold, spectra recorded in 100-mm cells. Broken curve is spectra of 0.75 M PrNDH in 0.1 mm cells. The contribution of PrND$^+$-chloride to the absorbance of the solution was negligible. All dilutions were made in the dark immediately after the mixtures were prepared.

RESULTS AND DISCUSSION

The mixing of a 10^{-2} M colorless solution of PrND$^+$-chloride with a 10^{-2} M light-yellow solution of PrNDH gave a solution with little increase in color intensity. At higher concentrations (0.7–1.5 M), an instantaneous orange color occurred. In these solutions the spectra showed a definite broadening and distortion of the tail absorption band which on dilution was almost completely abolished (Fig. 1). Reaction mixtures which were left standing at room temperature for a few hours showed residual absorption bands of higher intensity. At lower concentrations (10^{-4} M) the spectra of the reaction mixture scanned from 240 to 400 mμ merely represented the sum of the two component spectra. In solutions containing only PrNDH or PrND$^+$-chloride the intensity and shape of the absorption band was unchanged upon dilution when recorded in absorption cells wherein the light-path length was proportionally increased to the dilution.

On paper chromatography only two spots were observed corresponding to PrND$^+$-chloride and PrNDH. In mixtures left for 3 or more days at 4° or for a few hours at room temperature it was possible to identify a third component (orange) with a mobility

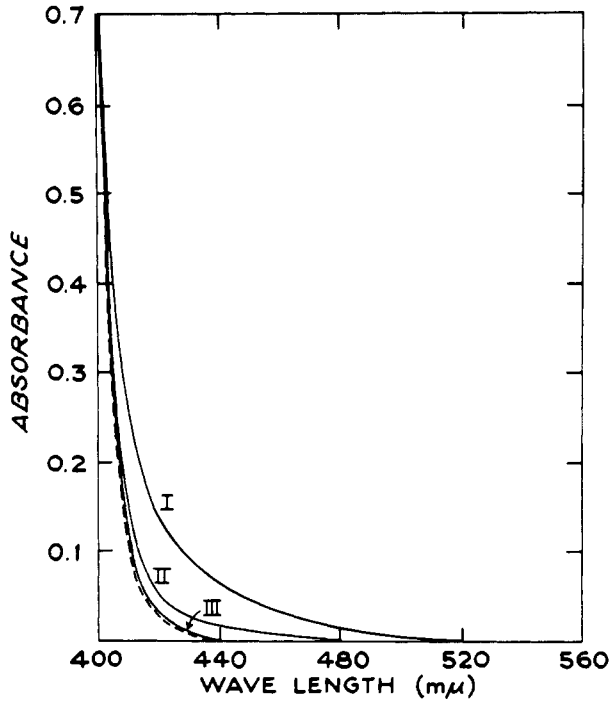

FIG. 2.— Dilution effect on the spectra of NAD$^+$ and NADH mixtures. I is 1.5 mmoles NADH and 1.5 mmoles NAD$^+$ in 10 ml of 0.1 M Tris, final *p*H 8.2. Spectra were immediately recorded in 1-mm cells. II is I after 10-fold dilution, spectra recorded in 10-mm cells; III is I after 100-fold dilution, spectra recorded in 100-mm cells. Broken curve is the spectrum of 0.075 M NADH in 1-mm cells. The contribution of NAD$^+$ to the absorption of the solution was negligible. All dilutions were made in the dark immediately after the mixtures were prepared.

falling between PrND$^+$-chloride and PrNDH. This compound, after elution with cold 70% 1-propanol, showed, however, only an absorption spectrum with max at about 300 mμ. It seems that the orange component was a degradation product from the standing of the reaction mixture, since the isolated components under similar conditions did not give the same orange compound. That the color of the reaction is dependent on the presence of the two components was shown by removing most of the PrNDH from the mixture with $CHCl_3$ extractions. The remaining H_2O layer was essentially colorless and contained PrND$^+$-chloride with little PrNDH. Similar spectrophotometric changes are found in the interaction of NAD$^+$ with NADH (Fig. 2). The high molecular weight and expense of the dinucleotides did not permit mixtures of higher concentration than 0.15 M for each component. Unlike the interaction of PrND$^+$-chloride with PrNDH, no residual band (degradation product) was found when fresh dinucleotide mixtures were diluted. The instability of these complexes was evident since, on dilution, spectrophotometric evidence of such interactions was no longer obtainable.

Electron paramagnetic resonance experiments in films obtained by the lyophilization of mixtures or by the mixing of reactant solutions using flow conditions and up to 0.75 M final concentration for each component were unsuccessful in detecting a free radical intermediate. The electron paramagnetic resonance spectrometer used was a Varian V-9502 adapted for both stopped and continuous-flow operations[4] (Yamazaki *et al.*, 1960).

[4] These experiments were performed in cooperation with L. Piette and W. Langford, Varian Associates, Palo Alto, Calif.

TABLE I
ISOTOPIC EXCHANGE BETWEEN NAD$^+$(T) AND NADH[a]

Expt.	Incubation Time (hr)	Products Analyzed (cpm/μmole) NAD$^+$ Nicotinamide	NADH Nicotinamide	NADH Ethanol[b]	Exchange (%)
A	0	44,000		264	
	4	35,700	3,760	4,670	19
	8	26,430	7,800	9,800	39
	12	19,800	11,500	14,200	57
	24	20,200	11,100	13,700	55
B	8	34,600	4,100	4,990	20
C	8		1,430	1,770	7

[a] In A, the reaction mixture contained 30 μmoles of NAD$^+$(T) (45,200 cpm/μmole) and 30 μmoles of NADH in a final volume of 1 ml of 0.1 M Tris, *p*H 8. Incubation temp, 30°. Keeping all conditions the same as A, expts B and C contained 3 and 0.3 μmoles of each dinucleotide, respectively, in a final volume of 1 ml. [b] Ethanol of lyophilization after NADH reoxidation with acetaldehyde and alcohol dehydrogenase, control corrected.

Figure 3 represents the isotopic exchange of the dinucleotide couples NAD$^+$-NADH and NADP$^+$-NADPH, as well as the isotopic exchange between the components of the PrNDH-(PrND$^+$-iodide) system. The results of these experiments are shown in Tables I to IV.[5] The direct tritium exchange between NAD$^+$ and NADH reached equilibrium in about 12 hours. The total specific activity of the isolated NADH (11,500 + 14,200) was about 57% the radioactivity of the NAD$^+$(T) (45,200), which was the starting radioactive component in this system (Table I, expt. A). This exchange also was observed in more dilute solutions, but at a slower rate (Table I, expts. B, C). Since the dinucleotide concentration level of these experiments (3×10^{-4} M) (Table I, expt. C) approaches the concentration commonly used in enzymic reactions, these results explain, in part, the lack of absolute asymmetry observed in enzymic reactions requiring long periods of incubation (Krakow *et al.*, 1963). Studies on mixtures containing NADP$^+$-(T) and NADPH showed that the equilibrium is reached at about the same time as in the NADH-NAD$^+$ system (Table II). About 3 hours of incubation was required, however, in the NAD$^+$-NADH(αT) couple (Table III). It appears that in this system the rate of exchange is dependent on the nature of the initial tritium-labeled component used in the incubation mixture. Also, it shows that the nonenzymic reduction of 3-acetylpyridine adenine dinucleotide by NADH(αD), an experiment described by Drysdale *et al.* (1961), was accompanied by an isotopic racemization involving no net oxidation-reduction.

Enzymic methods for demonstrating the distribution of tritium at C-4 of the nicotinamide ring were available only for the dinucleotide couples. The almost equal amount of tritium on both sides of C-4 of the nicotinamide ring established the symmetry of the isotopic exchange (Tables I and II). The isotopic exchange of the couples (PrND$^+$(T)-iodide)-PrNDH and (PrND$^+$-iodide)-PrNDH(T) is shown in Table

[5] In experiments where PrNDH and PrND$^+$-iodide were incubated for 12 hours, about 15% of the PrNDH was destroyed, with no apparent change in the concentration of the PrND$^+$-iodide component. In the dinucleotide systems the amount of oxidized dinucleotide hydrolyzed amounted to less than 15% in 12 hours, with slight decrease (ca. 4%) in concentration of NADH. Thus the concentration of the couple components does not necessarily remain constant throughout the incubation period.

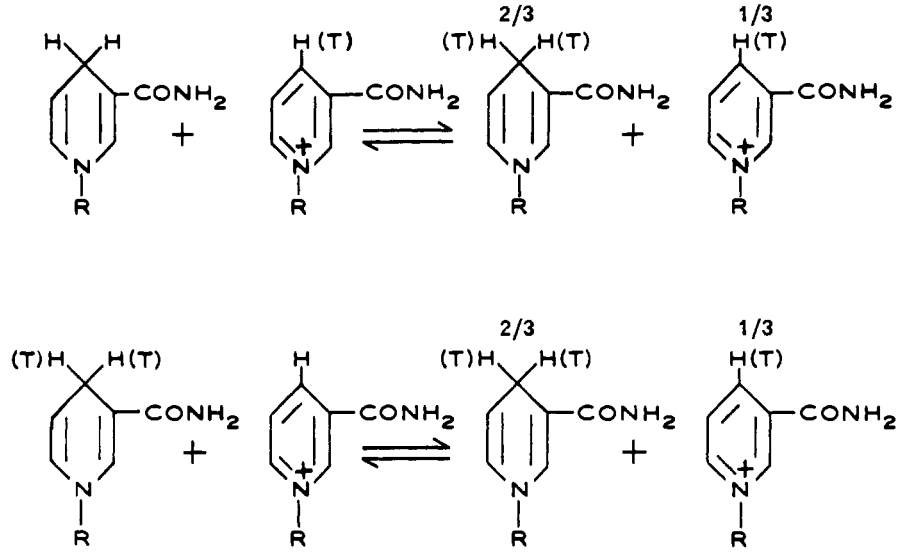

FIG. 3.—Isotopic exchange of 1-substituted nicotinamide couples. In each case, R represents n-C_3H_7 or the dinucleotide residues of NAD^+ or $NADP^+$. The anion group has been purposely omitted.

TABLE II

ISOTOPIC EXCHANGE BETWEEN $NADP^+$(T) AND NADPH[a]

Incubation time (hr)	Products Analyzed (cpm/μmole) NADP+ Nicotinamide	NADPH Nicotinamide	NADPH H_2O[b]	Exchange (%)
0	59,100		300	
1	53,000	2,800	3,200	10
4	46,500	5,800	7,200	22
8	34,000	12,600	14,100	45
12	23,000	16,600	19,000	60

[a] Reaction mixture contained 30 μmoles NADH and 30 μmoles of $NADP^+$(T) (60,000 cpm/μmole) in a final volume of 1 ml of 0.1 M Tris, pH 8. Incubation temp, 30°. [b] H_2O of lyophilization after NADPH reoxidation with GSSG and glutathione reductase, control corrected.

TABLE III

ISOTOPIC EXCHANGE BETWEEN NAD^+ AND NADH(αT)[a]

Expt	Incubation Time (hr)	Products Analyzed (cpm/μmole) NAD^+ Nicotinamide	NADH Nicotinamide	NADH Ethanol[b]	Exchange (%)
1	0		80	3770	
2	3	1270	212	2460	33
3	8	1350	566	1910	35

[a] Incubation mixture contained 30 μmoles of NADH(αT) (3870 cpm/μmole) and 30 μmoles of NAD^+ in a final volume of 1 ml of 0.1 M Tris, pH 8. Incubation temp, 30°. [b] Ethanol of lyophilization after NADH reoxidation with acetaldehyde and alcohol dehydrogenase, control corrected.

IV. In all these systems, regardless of the nature of the radioactive component, at equilibrium the amount of tritium on the reduced species was about two-thirds the total radioactivity of the reaction mixture. From statistical considerations, this is the amount of radioactivity which one would expect for a complete isotopic exchange between the couple components if such an exchange had proceeded through the formation of a complex intermediate.

The qualitative nature of the experiments reported in this paper does not demonstrate the detailed mechanism of hydrogen exchange. However, the direct hydrogen transfer and symmetry of the reaction seem to favor an ionic process involving a hydride exchange. So far, electron paramagnetic resonance does not support an alternate mechanism in which the hydrogen exchange takes place in two successive electron steps, free radical intermediates.

TABLE IV

ISOTOPIC EXCHANGE BETWEEN PrND+-IODIDE AND PrNDH

Expt	Incubation Time (hr)	Products Analyzed (cpm/μmole) PrND+-Iodide	PrNDH	Exchange (%)
A[a]	0	2510	90	3
	1.5	1070	1600	60
	3.0	975	1680	63
	4.5	780	1960	74
	6.0	845	1800	68
B[b]	0	0	654	0
	1.0	190	520	28
	2.0	151	497	23
	3.0	184	483	28
	9.0	190	418	32

[a] Reaction mixture contained 80 μmoles of PrND+(T)-iodide (2680 cpm/μmole) and 80 μmoles of PrNDH in a final volume of 2 ml of 0.1 M Tris, pH 10. Incubation temp, 30°. [b] Reaction mixture contained 80 μmoles of PrNDH(T) (680 cpm/μmole) and 80 μmoles of PrND+-iodide in a final volume of 2 ml of 0.1 M Tris, pH 10. Incubation temp, 30°.

Mixtures containing the couples NAD^+-NADH and (PrND+-chloride)-PrNDH were expected to interact with each other on the basis of the tendency of NAD^+ to accept electrons and NADH to give them up (Pullman and Pullman, 1959). Spectrophotometric studies presented here suggest that such interaction probably proceeds through the formation of a charge transfer complex as described in the theory of Mulliken (Mulliken, 1952; Kosower, 1960; Kosower, 1962; Szent-Gyorgi, 1960). A similar type of complex seems to be formed by mixing solutions of tryptophan or indolic compounds with NAD^+ and other 1-substituted nicotinamide derivatives (Cilento and Giusti, 1959; Alivisatos *et al.*, 1961). These systems, however, do not exchange hydrogen directly between the two components (unpublished experiments by the authors).

Thus it is the interesting feature of the dinucleotide and alkylnicotinamide couples that there is an obliga-

tory hydrogen exchange through the formation of an intermediate complex. However, there is no evidence as yet to suggest that the isotopic exchange process and the appearance of what may be a charge transfer complex are dependent upon each other. A similar phenomenon seems plausible also in the oxidation of NADH by its 3-acetylpyridine analog, observed by Spiegel and Drysdale (1960), wherein a pale-yellow solution mixture changes to orange upon freezing.

Acknowledgment

The authors gratefully acknowledge the advice of B. Vennesland and L. Strait and the facilities offered by the Department of Biochemistry and the School of Pharmacy, University of California Medical Center.

References

Alivisatos, S. G. A., Ungar, F., Jibril, A., and Mourkides, G. A. (1961), *Biochim. Biophys. Acta 51*, 361.

Anderson, A. G., Jr., and Berkelhammer, G. (1958), *J. Am. Chem. Soc. 80*, 992.

Cilento, G. (1960), *Arch. Biochem. Biophys. 88*, 352.

Cilento, G., and Giusti, P. (1959), *J. Am. Chem. Soc. 81*, 3801.

Colowick, S. P., Kaplan, N. O., Neufeld, E. F., and Ciotti, M. M. (1952), *J. Biol. Chem. 195*, 95.

Decker, R. H., Yess, N., and Brown, R. R. (1962), *Anal. Biochem. 3*, 357.

Drysdale, G. R., Spiegel, M. J., and Strittmatter, P. (1961), *J. Biol. Chem. 236*, 2323.

Fernandez, V. P., Mahler, H. R., and Shiner, V. J., Jr. (1962), *Biochemistry 1*, 259.

Fisher, H. F., Conn, E. E., Vennesland, B., and Westheimer, F. H. (1953), *J. Biol. Chem. 202*, 687.

Kaplan, N. O. (1955), *Methods Enzymol. 2*, 664.

Kaplan, N. O. (1961), *in* Symposium on Mechanism of Action of Steroid Hormones, Villee, C. A., and Engel, L. L., eds., New York, Pergamon, p. 247.

Karrer, P., Ringier, B. H., Büchi, J., Fritzsche, H., and Solmssen, U. (1937), *Helv. Chim. Acta 20*, 55.

Karrer, P., Schwarzenbach, G., Benz, F., and Solmssen, U. (1936), *Helv. Chim. Acta 19*, 811.

Karrer, P., and Stare, F. J. (1937), *Helv. Chim. Acta 20*, 418.

Kosower, E. M. (1960), *Enzymes 3*, 171.

Kosower, E. M. (1962), Molecular Biochemistry, New York, McGraw-Hill, p. 166.

Kosower, E. M., Martin, R. L., and Meloche, V. W. (1957), *J. Chem. Phys. 26*, 1353.

Krakow, G., Ludowieg, J., Mather, J. H., Normore, W. M., Tosi, L., Udaka, S., and Vennesland, B. (1963), *Biochemistry 2*, 1009.

LePage, G. A., and Mueller, G. C. (1949), *J. Biol. Chem. 180*, 975.

Loewus, F. A., Westheimer, F. H., and Vennesland, B. (1953), *J. Am. Chem. Soc. 75*, 5018.

Ludowieg, J., and Levy, A. (1962), *Fed. Proc. 21*, 239.

Ludowieg, J., and Levy, A. (1963), *Biochem. Biophys. Res. Commun. 11*, 19.

Marcus, A., Vennesland, B., and Stern, J. R. (1958), *J. Biol. Chem. 233*, 722.

Mulliken, R. S. (1952), *J. Am. Chem. Soc. 74*, 811.

Pastore, E. J., and Friedkin, M. (1961), *J. Biol. Chem. 236*, 2314.

Pullman, B., and Pullman, A. (1959), *Proc. Nat. Acad. Sci. U. S. 45*, 136.

Pullman, M. E., San Pietro, A., and Colowick, S. P. (1954), *J. Biol. Chem. 206*, 129.

San Pietro, A. (1955), *J. Biol. Chem. 217*, 579.

San Pietro, A., Kaplan, N. O., and Colowick, S. P. (1955), *J. Biol. Chem. 212*, 941.

Smith, M., and Symons, M. C. R. (1956), *J. Chem. Phys. 25*, 1074.

Spiegel, M. J., and Drysdale, G. R. (1960), *J. Biol. Chem. 235*, 2498.

Stern, B. K., and Vennesland, B. (1960), *J. Biol. Chem. 235*, 205.

Szent-Györgyi, A. (1960), Introduction to a Submolecular Biology, New York, Academic, p. 67.

Yamazaki, I., Mason, H. S., and Piette, L. (1960), *J. Biol. Chem. 235*, 2444.