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The Stability of Pyridine Nucleotides*

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Diphosphopyridine nucleotide and triphosphopyridine nucleotide when used in appropriate enzyme systems become exceedingly useful reagents for the measurement of almost any biochemical substance. A companion paper describes procedures for measuring each of the pyridine nucleotides, whether in the oxidized or reduced form, at concentrations as low as 10^{-9} M and in amounts as small as 10^{-15} moles (1). To exploit this sensitivity it was necessary to know more about the stability of the oxidized and reduced forms of the two nucleotides under a variety of conditions.

It is well known that the reduced pyridine coenzymes may be destroyed by acid without damaging the oxidized forms (2, 3) and that, conversely, the oxidized nucleotides may be destroyed by alkali without the slightest loss of the reduced forms (3–6). This extreme difference in behavior is a key to their enormous analytical potential.

This paper is intended as a practical statement of the range of conditions of temperature and pH value tolerated by the acid and basic forms. It can serve as a guide either for the destruction of the unwanted forms or for the preservation and storage of the desired forms. A particular problem dealt with, which is relevant to tissue analysis, is how to destroy diphosphopyridine nucleotidase without serious loss of the oxidized coenzymes. A second special problem encountered is how to destroy oxidized nucleotides and interfering enzymes in alkali, when blood is present, without loss of reduced coenzymes.

STABILITY OF DPNH AND TPNH

Acid Solutions—DPNH and TPNH are rapidly destroyed in acid (2, 3). The rate appears to be a linear function of hydrogen ion concentration (Fig. 1). At 23° the first order rate constant for DPNH is approximately equal to 380 (H^+) min^{-1} (7). This means that 99% of a reduced nucleotide would be destroyed in 0.6 minute in 0.02 N HCl. Fig. 1 permits calculation of rates of destruction in the range from pH 2 to 6. At pH 7 the calculated rate of destruction at 23° is 0.2% per hour. The rates of DPNH destruction at 38°, measured at pH 4.2 and 5.3 (acetate) and pH 7.1 (maleate), give a close fit to the line $k = 1380\ (H^+)$ per minute or a rate 3.6 times that at 23°. At 60° (Fig. 1) the rate is 6.0 times faster than at 38°. These results indicate a temperature coefficient of 9% per degree from 23–60°. TPNH has not been studied as extensively as DPNH but at 30° (pH 2 to 4.5) it was found to be destroyed 80% faster than DPNH.

As noted by Winer and Schwert (8), phosphate accelerates destruction of DPNH. At a phosphate concentration of 0.1 M the destruction rates were increased 3.5-fold at pH 6.2, and 7-fold at pH 6.8 whether the temperature was 25, 38, or 60°. EDTA (1 mM) had little effect on this phosphate acceleration. The enhanced loss of DPNH is not due to oxidation to DPN^+.

Alkaline Solutions and Optimal Storage Conditions—DPNH and TPNH are very stable in alkali. Warburg, Christian, and Griese (3) found no loss of TPNH after heating for 1 hour at 100° in 0.1 N NaOH. Adler, Hellström, and von Euler (6) found no destruction of DPNH after 30 minutes under the same conditions. This stability has been amply confirmed.

Although directly stable to alkali, DPNH and TPNH tend to become oxidized after long periods of time. DPNH was stored for nearly 3 months in an effort to find optimal storage conditions (Table I). The assays were based on the decrease in fluorescence produced by lactic dehydrogenase and pyruvate (0.2 mM) in 0.05 M phosphate buffer at pH 7. Before storage the DPNH preparation had residual fluorescence (after enzymatic oxidation) equal to 4% of the total fluorescence. This residual fluorescence increased in all samples in which there was loss on storage. This suggests that loss was due to oxidation to DPN^+ with subsequent conversion to its fluorescent derivative by alkali. It is also possible to demonstrate the accumulation of DPN^+ itself after prolonged storage at pH 10 (4°).

There appeared to be no loss under any of the conditions given in Table I at −85° (not shown). It will be seen that strong solutions of DPNH (40 mM) kept reasonably well at −20° at all pH values tested but underwent large losses at 4°, particularly at the more alkaline pH values. Conversely, weak DPNH solutions (0.4 mM) were rather stable at 4° at pH 9 to 11, but were destroyed to a considerable degree at −20° at pH 10.5 and above. The results can be explained by the assumption that impurities in the DPNH preparation or, conceivably, the DPNH itself, catalyze the oxidation especially at more alkaline pH values. This would account for greater loss in stronger samples at 4°. At −20°, impurities would be very concentrated in the residual liquid phase, and would accelerate oxidation of weak DPNH samples, but strong samples would be protected by precipitation of much of the DPNH. At −85°, there should be no liquid phase at all, and, consequently, no chance for catalyzed oxidation.

TPNH was not tested as extensively as DPNH, but a dilute solution (0.5 mM) in 0.02 N NaOH showed no loss in a week at 4° or −85°, but a 13% loss at −20°. The assays were based on decrease in fluorescence on the addition of α-ketoglutarate, NH_4^+, and glutamic dehydrogenase, and the subsequent reappearance of fluorescence after the addition of glucose-6-P and glucose-6-P dehydrogenase.

When the presence of oxidized nucleotides is to be avoided as far as possible, it would seem reasonable to recommend storage of reduced pyridine coenzymes at pH 10, *e.g.* in 0.05 M $NaHCO_3$-

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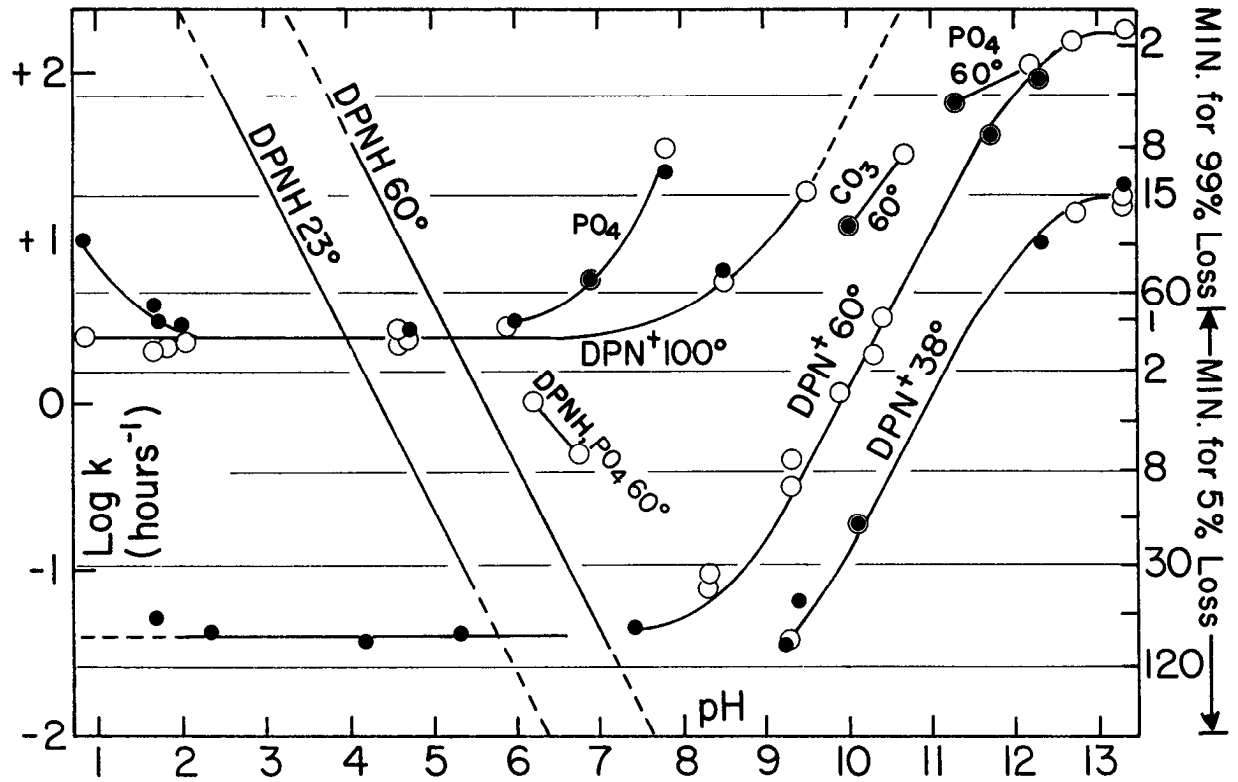

FIG. 1. Rates of destruction of DPN and DPNH as a function of pH value and temperature. The pH values were all measured at 25° with no attempt to evaluate the true values at the higher temperatures. The rates are plotted as the log of the first order reaction constants expressed as hours^{-1}. Thus, values with log k equal to -1 or 0 would indicate, respectively, initial rates of destruction of 10% and 100% per hour, or half-times of destruction of 6.9 and 0.69 hours. The lines for DPNH destruction are calculated from observations which clearly indicate a direct relationship between k and H^+ except in the presence of phosphate (see text). For the DPN^+ observations, solutions with pH values below 3 contained sulfuric acid or sulfuric acid plus sodium sulfate, except for one sample at 60° and at 100° (pH 1.7) which contained HCl (0.02 N). Unless otherwise indicated on the figure buffers for the rest of the samples were acetate, 0.04 to 0.2 M, at pH 4.0 to 5.2; Tris, 0.1 M, at pH 7.3 to 8.3; 2-NH_2-2-methyl-1, 3-propanediol, at pH 8.3 to 9.3; and 2-NH_2-2-methyl-1-propanol at pH 9.3 to 10.5. NaOH was used above pH 11.5. Samples indicated by *open circles* were analyzed by fluorescence produced in strong alkali; those indicated by *solid circles* were analyzed enzymatically (see text). The curve for destruction of DPN^+ at 60° is calculated from the formula $k = 0.04 + 185\,B$, where B (basic form) is expressed as the fraction of the total DPN^+. B is calculated from the Henderson-Hasselbalch equation assuming pK_a of 12.2 (see text).

0.05 M Na_2CO_3 at 4°, and at concentrations no greater than 5 mM. DPN^+ or TPN^+, if present originally, or slowly accumulating, may be removed by heating for 30 minutes at 60°, or 1 or 2 minutes at 100° (see below).

Effect of Blood—It was found that blood, even at high dilution, is capable of greatly accelerating DPNH and TPNH oxidation in alkaline solution. For example, at 25° in 0.02 N NaOH, rat blood at concentrations of 0.02, 0.01, 0.005, and 0.0025% oxidized, respectively, 46, 39, 24, and 19% of DPNH present (10^{-6} M). The percentage rate of loss was approximately twice as great with 4×10^{-7} M as with 5×10^{-6} M DPNH. Thus, the effect is greater with more dilute DPNH and stronger concentrations of blood, but not in direct proportion. Blood at 1% concentration is very little more destructive than at 0.2%. The loss occurs more rapidly at 60 and 100 than at 25°, but the total loss may be no greater, since during heating (*e.g.* 10 minutes at 60° in 0.02 N NaOH) the blood loses its capacity to oxidize DPNH. The loss increases somewhat with the strength of alkali, whereas at pH 10.0 to 10.7 (carbonate) there is little or no loss in 10 minutes at 60° There is no sign that the effect is enzymatic. The addition of DPN^+ does not prevent the DPNH loss.

This destructive property of blood presents a serious problem in the assay for DPNH and TPNH in tissues, since small amounts of blood are nearly always present. A wide variety of substances were tried in an effort to prevent the DPNH loss. Little or no protection was obtained with nicotinamide (20 mM), $NaNO_2$ (10 mM), NaSCN (50 mM), Na_2SO_3 (50 mM), Na_2S (5 mM), sodium Amytal (10 mM), $MgCl_2$ (3 mM), or NaCN (1 mM). EDTA (1 mM) may be partially protective. Protection was, however, obtained with 0.1% bovine plasma albumin. The individual amino acids were tested at the concentrations present in 0.1% bovine plasma albumin. Tryptophan and cysteine were protective but no other amino acid present in plasma albumin, including cystine, had any effect. DPNH (10^{-6} M) in 0.02 N NaOH was completely protected against 0.02% blood by 6×10^{-6} M cysteine or 5×10^{-5} M tryptophan. Mercaptoethanol at 10^{-4} M also protects under these conditions, but has not been extensively tested. Neither cysteine (up to 3 mM) nor tryptophan (up to 2 mM) is protective to DPNH in strong blood concentrations (1%), but cysteine (0.5 mM) will protect 10^{-5} M DPNH from destruction in 0.2% blood.

As a result of these findings, it is recommended that tissues being prepared for TPNH or DPNH assay be homogenized in

TABLE I

Storage of DPNH for long periods

DPNH at two concentrations was stored under conditions indicated. The amount remaining is recorded as a percentage. The solutions were analyzed enzymatically (see text). The media were, respectively, 2-NH_2-2-methyl-1,3-propanediol buffer, 0.1 M; Na_2CO_3, 0.075 M; $NaHCO_3$, 0.025 M; Na_3PO_4, 0.04 M; K_2HPO_4, 0.04 M; and NaOH, 0.05 M.

pH	Storage time	DPNH, 0.4 mM		DPNH, 40 mM	
		4°	−20°	4°	−20°
	days				
9.1	16		101	97	96
	80	92	90	75	86
10.5	16	100	97	96	101
	80	89	74	66	93
11.2	16	97	80	92	104
	80	91	57	35	91
12.7	16	86	28	72	96
	80	74	8	29	88

ice-cold 0.02 N NaOH containing 0.5 mM cysteine and then heated at once for 10 minutes at 60°. This will destroy TPN^+, DPN^+, and interfering enzymes. Cysteine (added as the hydrochloride) is sufficiently stable for several hours at 0° in 0.02 N NaOH to remain effective. Tryptophan heated with blood in alkali gives a strongly fluorescent product and, therefore, has not been further explored. It is recommended that blood itself be prepared for assay by heating at a dilution of 1:100 for 10 minutes at 60° in 0.1 M Na_2CO_3-0.02 M $NaHCO_3$ (pH 10.7). This weaker alkaline treatment is not safe to use with tissues, since at pH 10.7, liver, at least, rapidly oxidizes a substantial fraction of added DPNH even at 0°.

In experiments reported separately (1) the validity of the recommended procedures was confirmed by showing satisfactory recovery of DPNH and TPNH from homogenates of brain and liver and from diluted blood (rat).

STABILITY OF DPN^+ AND TPN^+

Effect of pH Value—Alkali accelerates the rate of destruction of DPN^+ and TPN^+ (4) but there is not a direct relationship between OH^- and rate except between pH 9 and pH 11.5 (Fig. 1). The results are those predicted if the molecule has a low constant rate of destruction from pH 2 to 7, and is converted by removal of 1 proton to a form several thousand times more susceptible to destruction (see legend of Fig. 1).

The temperature coefficient for destruction of DPN^+ is 11% per degree between 60 and 100° from pH 1.7 to 9.5, and also 11% per degree between 38 and 60° from pH 9.5 to 13.

The destruction rate is increased by a number of salts. At pH 10 and 60°, the rate was found to be increased 40 to 100% by NaCl, sodium oxalate, Na_2SO_4, Na_2HPO_4, and sodium maleate (in this order) when these were added at 0.2 M concentration to a 0.1 M buffer of 2-amino-2-methyl-1-propanol. Carbonate has a greater effect (Fig. 1). When added to the same buffer as the other salts the rate of destruction was increased 380, 500, and 630% at 60° by 0.1 M, 0.2 M, and 0.5 M carbonate buffer at pH 10 (a 1:1 ratio of $NaHCO_3$ and Na_2CO_3). The acceleration was not affected by adding EDTA at 1 mM. Colowick, Kaplan, and Ciotti (9) found that phosphate accelerates DPN^+ destruction in the neighborhood of pH 7. This has been confirmed (Fig. 1). At pH 7.8 the acceleration by 0.1 M phosphate was reduced from 8-fold to 5-fold by adding EDTA at a concentration of 1 mM. Colowick *et al.* observed that Tris buffer will almost abolish the phosphate acceleration. The earliest studies of the effect of pH value on DPN^+ destruction (10) were made in phosphate in the range of pH 4.6 to 7, with results that resemble those of Fig. 1.

At all pH values higher than 2 there was close correspondence between DPN^+ assayed by the fluorescence produced in 6 N NaOH (11), and DPN^+ assayed enzymatically (Fig. 1). Presumably, destruction above pH 2 is the result of cleavage of nicotinamide from the molecule, as has been shown to be the first step in degradation in alkali (11, 12) or at neutrality (9). At pH values lower than 2 destruction measured enzymatically exceeds that measured chemically. The difference is probably due to hydrolysis of the pyrophosphate bond. The enzyme assays for the samples treated at 100° were made by reducing the DPN^+ with 50 mM α-glycerol-P and glycerol-P dehydrogenase at pH 9.2 and measuring the fluorescence of the DPNH formed. The samples at 60 and 38° were similarly measured by reduction with 5 mM glutamate and glutamic dehydrogenase.

Samples of TPN^+ and DPN^+ (0.5 mm) were stored for a week at 4, −20, and −85°, in 0.02 N HCl. No loss was detected at 4° or −85° in either case, but both coenzymes were destroyed to the extent of approximately 15% at −20°. Presumably, at −20° there remains a small amount of liquid phase with a sufficiently high concentration of HCl to hydrolyze the nucleotides. The assays were conducted enzymatically. TPN was reduced with glucose-6-P dehydrogenase and a moderate excess of glucose-6-P. After measuring the fluorescence, α-ketoglutarate, NH_4^+, and glutamic dehydrogenase were added to reoxidize the coenzyme. Since the fluorescence changes in both directions agreed, it is concluded that there is little danger of conversion of TPN^+ to DPN^+ by storage in weak acid.[1] Similarly, DPN^+ was first reduced with α-glycerol-P and glycerol-P dehydrogenase, as above, and reoxidized (after heating to 100°) with glutamic dehydrogenase (as for TPN^+). Again, measurements in both directions agreed.

Prevention of Enzymatic Destruction of DPN^+ and TPN^+—To prepare tissues for the measurement of DPN^+ and TPN^+ it is difficult to destroy the enzyme which cleaves nicotinamide from DPN^+ or TPN^+ without destroying the nucleotides as well. This DPNase is actually more stable to heat below pH 2 than it is in weaker acid or near neutrality. Rabbit brain homogenate (1:100), when heated in 0.02 N HCl for as long as possible without destroying more than 5% of DPN^+ present (50 minutes at 60°; 15 minutes at 75°; 3 minutes at 90°), retained more than half the enzyme activity. Even exposure to 0.1 N and 0.2 N HCl for 10 minutes at 60° left much of the activity. The enzyme could be destroyed at pH 3.7 (formate) and pH 4.7 (acetate) by heat treatment mild enough not to destroy DPN^+. However, the enzyme showed substantial activity at these pH

[1] A more rigorous test of this point was made by heating TPN^+ for 30 minutes in 0.02 N H_2SO_4 at 60°. NO DPN^+ formation was observed with an enzymatic dismutation system (1) that would have detected the presence of 1 part of DPN^+ in 2000 parts of TPN^+.

values. Consequently, there might be losses during preparation of homogenates in either formate or acetate buffer.

Fortunately, between pH 2.2 and 2.6 (H_2SO_4 plus Na_2SO_4) the enzyme is not active and 95% of the enzyme can be destroyed without more than 3% loss of DPN^+ or TPN^+. The recommended procedure is to homogenize in ice-cold 0.02 N H_2SO_4-0.1 M Na_2SO_4 (pH 2.3) and then heat 45 minutes at 60°. Although some activity remains, it is not enough to matter with tissue diluted several thousandfold under the conditions for measurement of pyridine nucleotides by cycling procedures (1). In case this degree of dilution is not possible, the material, after preliminary heating at pH 2.3, could be brought to pH 5 to 7 and immediately reheated for 3 minutes at 90° to destroy residual enzyme.

SUMMARY

1. The stability of oxidized diphosphopyridine nucleotide has been determined from pH 1 to 13 at temperatures ranging from 25–100°.

2. The stability of reduced diphosphopyridine nucleotide has been measured from pH 2 to 13 over the same temperature range.

3. Storage capabilities of the oxidized pyridine nucleotides in acid and the reduced forms in alkali have been assessed at 4, −20, and −85°.

4. The presence of blood, even at very high dilution, results n loss of reduced coenzymes during alkaline treatment to destroy the oxidized forms and interfering enzymes. Means to avoid this difficulty are described.

5. The enzyme which cleaves nicotinamide from oxidized pyridine nucleotides is difficult to destroy without serious loss of the nucleotides. A partial solution to this problem is presented.

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