THE FLUOROMETRIC MEASUREMENT OF PYRIDINE NUCLEOTIDES*

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Measurement of DPNH[1] or TPNH by absorption at 340 mμ has been a standard procedure for many years and has been used to study innumerable enzyme reactions. In spite of the proved value of this colorimetric method, there may be advantages in fluorometric methods for the pyridine nucleotides.

First, it has been found practicable with a fluorometer to determine separately oxidized and reduced pyridine nucleotides. As a result, the formation of a little DPN^+ from a large amount of DPNH may be easily measured, and vice versa. Second, each determination may be made accurately at a nucleotide concentration as low as 10^{-8} M, *i.e.* at a concentration only a thousandth of that required for ordinary colorimetry at 340 mμ. Third, the useful range of sensitivity is about 2000-fold, which is much greater than that obtained with colorimetry. Since fluorometry may be performed with as little as 0.01 ml. (2), it is possible to measure 10^{-13} mole of either oxidized or reduced pyridine nucleotide. Amounts of DPN^+ or DPNH as great as 10^{-11} mole or over may be measured rapidly and easily in a volume of 1 ml.

The fluorometric methods, because of their sensitivity, have been of considerable utility for quantitative histochemical purposes. With the addition of appropriate accessory enzymes, almost any enzyme reaction may be made to produce an oxidation of DPNH or TPNH or a reduction of DPN^+ or TPN^+.

The basic method for DPN^+ (TPN^+) is that of Kaplan *et al.* (3). The method has merely been changed slightly to increase reproducibility and to provide for destruction of DPNH which otherwise contributes some fluorescence. The method has also been studied further to establish some

* A preliminary report of this work has been published (1).

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[1] The abbreviations used include pyridine nucleotide, PN; oxidized and reduced diphosphopyridine nucleotides and triphosphopyridine nucleotides, respectively, DPN^+ and DPNH, TPN^+ and TPNH; tris(hydroxymethyl)aminomethane, Tris.

of its limitations. To measure DPNH (TPNH), DPN^+ is destroyed in weak alkali. After this, in one analytical step, DPNH is (*a*) oxidized to DPN^+ and (*b*) converted to the same fluorescent product as that used to measure preformed DPN^+. If 10-fold less sensitivity is sufficient, the well known native fluorescence of DPNH and TPNH is used for their measurement. Theorell *et al.* have recently exploited this native fluorescence in kinetic studies at low DPNH levels (4). Some unexpected effects of cations on the fluorescence will be described.

The use of these procedures will be illustrated by (*a*) values for the lactic dehydrogenase and 6-phosphogluconate dehydrogenase activities of single large nerve cell bodies, (*b*) the Michaelis constant for TPN^+ with glucose-6-phosphate dehydrogenase of brain, a constant so low that it would be difficult to measure by absorption at 340 mμ, and (*c*) measurements of the concentrations of DPN^+, DPNH, TPN^+, and TPNH in brain and liver.

Procedures

DPN^+ (TPN^+)

The sample to be analyzed is made 0.2 N in HCl for at least 30 seconds to destroy DPNH. Strong NaOH is added to give a concentration of 6 N, and the sample is allowed to stand for 1 hour at room temperature or 30 minutes at 38°. It is then diluted at least 5-fold with water and read in a fluorometer with a primary filter of Corning glass No. 5860 and secondary filters of Corning Nos. 4308, 5562, and 3387.[2] The final nucleotide concentration may be from 10^{-8} to 2×10^{-5} M. Quinine solutions in 0.1 N H_2SO_4 (0.005 to 5 γ per ml.) serve as working fluorometer standards.

The absolute volumes are arbitrary. A typical example is as follows: A 5 μl. sample containing 10^{-11} to 10^{-8} mole of DPN^+ is added to 10 μl. of 0.3 N HCl. After mixing, a 10 μl. aliquot is transferred to a 3 ml. fluorometer tube containing 100 μl. of 7 N NaOH (prompt mixing). After an hour, 1 ml. of water is added, and the sample is ready to be read. It is necessary to provide standards and blanks which are given identical treatment.

When the quantity of DPN^+ to be measured is small in comparison to the amount of DPNH present, it is essential that none of the sample es-

[2] Any fluorometer with sensitivity sufficient for the particular purpose is satisfactory. For measuring the very lowest levels, the sensitive and convenient fluorometer model A of the Farrand Optical Company, Bronx, New York, has been entirely satisfactory. The primary filter prescribed isolates the Hg line at 365 mμ. The secondary filter complex has maximal transmission at 470 mμ and was chosen empirically to give a low relative blank value. The usual "B1" secondary filter is satisfactory with higher DPN^+ levels. It isolates a much broader spectral band and would give much greater sensitivity with some sacrifice of selectivity.

capes contact with HCl; otherwise results will be erratic from undestroyed DPNH.

DPNH (TPNH); Strong Alkali Method

The sample to be analyzed is made 0.02 to 0.04 N in NaOH (excess) and heated either for 15 minutes at 60° or for 2 hours at 38° to destroy DPN^+. (If desired, a 0.2 to 0.5 M buffer consisting of a 3:1 molar ratio of Na_3PO_4 to K_2HPO_4 can be used instead of the weak NaOH.) Strong alkali is next added to give a concentration of 6 N as when measuring DPN^+, except that the alkali contains H_2O_2 at a final concentration of about 0.01 per cent. The alkaline peroxide is prepared from aqueous 3 per cent H_2O_2, which in turn is prepared every week or so from 30 per cent H_2O_2. (A precipitate which is hard to dissolve may form on addition of 30 per cent H_2O_2 to strong alkali.) The alkaline peroxide solution is not very stable and is prepared within an hour of use if kept at room temperature or within a few hours if kept in ice water. After addition of this solution, the sample is allowed to stand for 60 minutes at 38° or 2 hours at room temperature and is then diluted at least 5-fold and read in the fluorometer.

The absolute volumes are arbitrary. A typical example is the following: A 0.1 ml. sample containing 2×10^{-10} to 10^{-7} mole of DPNH is added to 1 ml. of 0.04 N NaOH and heated for 15 minutes at 60°. A 50 μl. aliquot is transferred to a 3 ml. fluorometer tube containing 100 μl. of 0.015 per cent H_2O_2 in 9 N NaOH. After an hour, 1 ml. of water is added, and the sample is ready to be read.

DPNH (TPNH); Native Fluorescence Method

The sample to be analyzed is merely diluted to a concentration of 10^{-5} to 10^{-7} M with a solution having a pH between 8 and 12. It is usually convenient to stop an enzyme reaction by using 0.01 N NaOH as the diluent. If Mg is present at a final concentration greater than 10^{-5} M, and if the diluting fluid has a pH greater than 11, the dilution should be made with a fluid containing sodium Versenate (ethylenediaminetetraacetate) in excess of the Mg present (see below). Suitable blanks and standards are prepared and read at the same time.

Measurement of Native Pyridine Nucleotides in Tissues

Although the procedures given were not primarily designed for the measurement of naturally occurring pyridine nucleotides, they nevertheless seem suited to such measurements. Fig. 1 gives a practical scheme for a separate determination of DPN^+, TPN^+, DPNH, and TPNH. To measure only total oxidized PN and total reduced PN, the analysis need be carried no further than Solutions B and G (Fig. 1), and a blank is ob-

tained by treating Solution B with weak alkali so as to destroy oxidized PN. If it is wished to measure total DPN and total TPN separately, Extract A may be treated with a mixture of glucose-6-phosphate dehydrogenase and alcohol dehydrogenase (with glucose-6-phosphate and acetaldehyde), whereupon all the DPN will be oxidized and all the TPN reduced. Each may then be selectively destroyed with acid or alkali.

An actual procedure tested with brain and liver, which appears to be satisfactory for measuring all four forms of PN, is as follows: A glass homogenizer containing 0.05 M Tris buffer (3 ml. for brain, 8 ml. for liver)

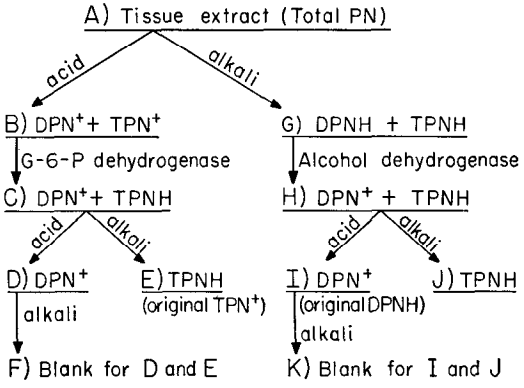

FIG. 1. Flow sheet for measuring pyridine nucleotides in tissues

was placed in a water bath at 85°. Tissue, weighing 0.3 gm., was added and mashed with a rod during 5 minutes of heating.[3] The quickly cooled sample was homogenized and centrifuged at 12,000 × *g* for 60 minutes (Extract A).

Aliquots of 500 μl. of Extract A were treated with either 30 μl. of 1 N HCl for a few minutes at room temperature or 30 μl. of 1 N NaOH for 15 minutes at 60°. Each sample was then restored to pH 8 by adding 30 μl. of 1 N NaOH or 30 μl. of 1 N HCl to give Solutions B and G (Fig. 1).

To Solution B (now 560 μl.) were added 6 μl. of 100 mM glucose-6-phosphate and 15 μl. of a glucose-6-phosphate dehydrogenase preparation from yeast[4] to give Solution C. The reaction was allowed to proceed at least

[3] The method of preparing the tissue extract is taken from Greengard *et al.* (5), who found pH 8.2 to be the safest pH for heating without undue destruction of either oxidized or reduced PN. These investigators heated samples for 15 seconds at 100°.

[4] This was kindly supplied by Dr. David H. Brown. It had an activity (measured in the spectrophotometer) of 240 moles per liter per hour (25°) when measured at pH 8 with 1 mM glucose-6-phosphate and 0.15 mM TPN^+. Thus the calculated V_{max} during enzyme action would be about 6 mmoles per liter per hour. Oxidation with

for 30 minutes at room temperature. Under these conditions, TPN^+ at a concentration of 0.022 mM was reduced 90 per cent in 4 minutes and nearly 100 per cent in 8 minutes. The Tris buffer concentration was kept low, since it is inhibitory at higher levels (75 per cent inhibition in 0.5 M Tris). Some yeast preparations of glucose-6-phosphate dehydrogenase contain a factor destructive to TPN^+. Therefore it is desirable to add the enzyme last. Isocitrate and its dehydrogenase might well be substituted for glucose-6-phosphate dehydrogenase.

Aliquots of Solution C, 30 μl. each, were treated with either 2 μl. of 1 N HCl (briefly) or 2 μl. of 1 N NaOH (15 minutes at 60°) to give Solutions D and E, respectively. Additional samples equivalent to Solution D were further treated with 2 μl. of 2 N NaOH at 60° for 15 minutes to give Solution F. Aliquots of Solutions D, E, and F of 25 μl. were added to 100 μl. volumes of 8 N NaOH containing 0.01 per cent H_2O_2 in 3 ml. fluorometer tubes. After 60 minutes at 38°, 1 ml. of water was added to each, and the fluorescences of these samples and of suitable standards were measured.

Solution G (volume 560 μl.) was treated with 8 μl. of 0.4 per cent acetaldehyde, 2 μl. of a 1:10 dilution of yeast alcohol dehydrogenase solution (Sigma Chemical Company, St. Louis), and 13 μl. of 35 mM sodium Versenate to give Solution H. The reaction was allowed to proceed for 30 minutes at room temperature. Under these conditions DPNH at a concentration of 0.023 mM was oxidized almost 100 per cent in less than 1 minute. The Versene was added to protect alcohol dehydrogenase against possible traces of heavy metals. The concentration of Tris is kept low, since yeast alcohol dehydrogenase was found to be especially sensitive to inhibition by high concentrations of this buffer. (The activity with 0.11 mM DPNH was only 24 and 9 per cent as great in 0.2 and 0.5 M Tris as it was in 0.05 M. Tris hydrochloride was used, but the effects of chloride ion described by Theorell *et al.* (4) do not explain this inhibition. NaCl at a concentration of 0.5 M inhibited only 45 per cent.)

After incubation, aliquots of Solution H were treated as described for Solution C, and the resulting Solutions I, J, and K were analyzed in the same manner as Solutions D, E, and F.

EXPERIMENTAL

Fluorescence from DPN^+

Formation of Fluorescence and Stability—As Kaplan *et al.* observed (3), DPN^+ is destroyed by weak alkali with little development of fluorescence, but in strong alkali the destruction results in the formation of a fluorescent

DPN^+ was not detected, and, if present, must have been at least 1000 times slower than with TPN^+.

product. These authors found that nicotinamide riboside reacted in the same manner, but that N^1-methylnicotinamide gave only 1.4 per cent as much fluorescence as DPN^+, while nicotinamide did not develop fluorescence. The DPN^+ product has an absorption maximum at 360 mμ. The fluorescence, once developed, is constant from pH 10.5 to 15. It decreases to zero at pH 6, with a 50 per cent value at pH 9.6 (3).

The fluorescence from DPN^+ increases almost linearly with NaOH concentration from 0.05 to 6 N NaOH. The situation is explicable on the basis of two competing reactions, (1) conversion of DPN^+ to a non-fluorescent product by a reaction which is not accelerated by NaOH stronger than 0.05 N and (2) conversion of DPN^+ to a fluorescent product by a reaction with velocity proportional to NaOH concentration. The rate of fluorescence development, as this predicts, slows down faster than for an uncomplicated monomolecular reaction. The two competing reactions must have almost equal temperature coefficients, since maximal fluorescence was found to be the same within 1 per cent at 38°, 45°, or 60°. Once fluorescence is developed, it is quite stable except under strong illumination. Even heating for 30 minutes at 60° in 6 N NaOH did not affect the fluorescence. Approximately 10 per cent more fluorescence is produced in 8 N NaOH than in 6 N, but increasing difficulties due to viscosity recommend the lower level of alkali.

In 6 N NaOH the fluorescent product is sufficiently sensitive to destruction by ultraviolet light to make readings a little difficult when using a strong light source. Fortunately light sensitivity is amazingly diminished by dilution (Fig. 2). The sensitivity is 50 times less in 1 N than in 10 N NaOH. Presumably the light-susceptible molecules represent a small minority of the total, and they increase almost as the square of the NaOH concentration. Below 1 N NaOH there is no further decrease in light sensitivity.

Elimination of DPNH Interference—The native fluorescence of DPNH is about 10 per cent of that of the inducible fluorescence for the same amount of DPN^+. The fluorescence of DPNH has a broader wave band than that of the DPN^+ product (Fig. 3); hence, the ratio of DPNH fluorescence to inducible DPN^+ fluorescence varies with the wave length at which it is measured. If DPNH is treated with 6 N alkali, fluorescence as great as a quarter of that from DPN^+ may be obtained, apparently due to partial oxidation of DPNH to DPN^+.

Both native and inducible fluorescence from DPNH are completely destroyed by momentary acidification. The rate of acid destruction of DPNH is first order with observed half time of 49 minutes at pH 4.4 and 0.43 minute at pH 2.4 (23°). Destruction is thus proportional to (H^+) and would be 99 per cent complete in about 10 seconds in 0.1 N HCl. Disap-

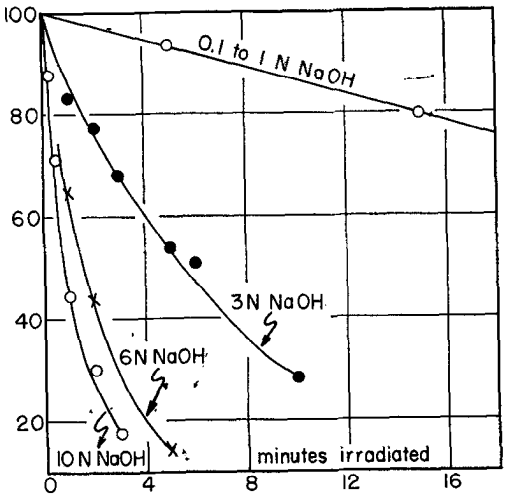

FIG. 2. Light sensitivity of fluorescent DPNH product in NaOH of different strengths as shown. The ordinate is the per cent of initial fluorescence remaining after irradiation for the time indicated. The half times for the four curves are 0.9, 1.6, 5.8, and 45 minutes. Samples were irradiated at a distance of about 6 cm. from the arc of a BH4 mercury arc lamp (General Electric Company, Schenectady).

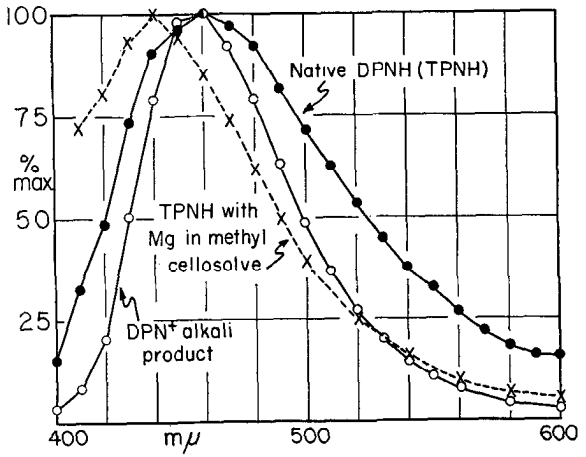

FIG. 3. Fluorescent spectra of DPNH in water, of TPNH in methyl Cellosolve with 1 mM Mg, and of the fluorescent product obtained from DPN^+ with strong alkali. The spectra were obtained on the Beckman spectrophotometer as previously described (6). TPNH and TPN^+ gave spectra which were indistinguishable from those for DPNH and DPN^+. It should be noted that values are plotted as per cent of the maximum. On an absolute scale, the DPNH curve would have to be reduced by a factor of about 8.

pearance of DPNH fluorescence (read in weak alkali) was parallel to disappearance of light absorption at 340 mμ.

As expected, if nicotinamide riboside is exactly as fluorogenic as DPN^+, prolonged standing in acid does not appreciably change the amount of fluorescence subsequently produced from DPN^+ or TPN^+. After 1, 20, 120, and 1000 minutes in 0.2 N HCl, the fluorescences from DPN^+ had relative values of 1.00, 1.01, 1.02, and 1.03, and those from TPN^+ had relative values of 1.00, 1.00, 1.02, and 1.01. (This is also indirect proof that DPN^+ and TPN^+ give the same amount of fluorescence.)

The residual fluorescence obtained with a DPNH solution after acid treatment varies with the particular preparation and appears to be due to small amounts of DPN^+ present. In addition, during storage in solution

TABLE I

Oxidation of DPNH during Storage

A sample of DPNH "90" of the Sigma Chemical Company was analyzed for DPN^+ by the present procedure after storage under the conditions shown. The values are recorded for DPN^+ as per cent of the DPNH initially present.

Solvent	Initial	24 hrs.			96 hrs.	
		25°	4°	−20°	4°	−20°
pH 7.1, 0.1 M phosphate.....	1.6	6.9	2.0	2.9		
" 8.7, 0.1 " Tris.....	1.4	2.5	1.3	1.3	1.9	1.7
" 9.9, 0.1 " AMP*.....	1.3	2.6	1.6	1.6	2.0	1.8

* 2-Amino-2-methylpropanol.

DPNH is oxidized at varying rates, depending upon temperature and pH of the solution (Table I). This is of practical concern in keeping the blank value as low as possible when DPN^+ formation is being used to measure a reaction. Storage at pH 9 in Tris buffer is recommended.

Proportionality and Reproducibility—Fluorescence is proportional to concentrations between 3×10^{-9} and 3×10^{-6} M (Table II). At higher concentrations readings are no longer proportional because a significant fraction of the exciting light is absorbed within the sample so that deeper layers are not fully illuminated.

Reproducibility is largely a matter of stability of the fluorometer and to a lesser extent the care with which the fluorometer tubes have been selected rather than the reproducibility of the fluorogenic reaction itself. A coefficient of variation of 1 or 2 per cent is easily attainable at all but the lowest levels. The precision is also less if the fluorescence blank is relatively large from whatever cause. As with any fluorometric measurement, the temperature of sample and standard must be the same because of the high nega-

tive temperature coefficient (in this case 1.3 per cent per degree between 20° and 38°).

Interfering Substances and Specificity—Most substances, in amounts likely to be encountered, do not disturb the measurement of DPN^+ or the disturbance may be eliminated by dilution. However, it is necessary to keep the concentration of certain reactive substances rather low at the stage of development of fluorescence in strong alkali. For example, pyruvate and α-ketoglutarate inhibit development of fluorescence at concentrations greater than 0.1 mM at this stage. When α-ketoglutarate was present at concentrations of 0.3, 1.0, and 3.5 mM, the readings with DPN^+ were

TABLE II

Proportionality between Fluorescence and Concentration

Measurements were made with 1 ml. in 10 × 75 mm. tubes. The concentrations are recorded as 10^{-7} M. The 10^{-8} M DPN^+ sample and the 3×10^{-7} M DPNH sample were used as reference standards for the rest of the samples.

DPN^+; induced fluorescence		DPNH; native fluorescence	
Concentration present	Calculated from fluorescence	Concentration present	Calculated from fluorescence
108	90	111	110
29.6	28.0	34.2	35.6
10.8	10.7	11.1	11.3
2.98	3.07	3.43	3.43
1.08	1.09	1.04	0.99
0.31	0.33		
0.112	0.112		
0.031	0.028		

diminished 15, 34, and 63 per cent, respectively. Oxalacetate and glucose inhibit if present much in excess of 1 mM during fluorescence development. The presence of 0.02 per cent peroxide in the strong alkali will reduce interference from all of these carbonyl compounds. In any event it is desirable to measure standards under conditions identical to unknown samples.

When DPNH is treated with acid in the presence of reduced cytochrome *c*, it appears to be oxidized to DPN^+. This does not occur with oxidized cytochrome *c*. The oxidation seems to be due to cytochrome *c* itself, since the same effect occurred whether the cytochrome was reduced by ascorbic acid, hydrosulfite, or cytochrome reductase.

Anything which absorbs light at 365 to 550 mμ will of course decrease the reading. This trouble is usually avoidable through dilution and by the use of an internal standard.

The specificity of the reaction for DPN^+ has not been extensively tested,

since the present use is chiefly for measuring oxidation of DPNH or TPNH or the cleavage of DPN^+ by DPNase. DPN^+ and TPN^+ give the same amount of fluorescence within a few per cent (based on assay of the nucleotide preparations with alcohol dehydrogenase and glucose-6-phosphate dehydrogenase, respectively). In boiled extracts of liver and brain, fluorescence from non-pyridine nucleotide substances does not exceed 10 per cent of that from total pyridine nucleotides present (see below).

Comparison with Ketone Method for DPN^+—DPN^+ may be measured by formation of products with acetone (7) or methyl ethyl ketone (8) which are strongly fluorescent in acid. The method of Kaplan *et al.* is almost as sensitive as the methyl ethyl ketone method; it is at least as specific and simpler and more reproducible. Particularly with very small volumes the use of volatile ketone is troublesome. The ketone method gives a 10-fold lower reading with DPNH than the strong alkali method and could therefore be used to measure DPN^+ without pretreatment with acid. After destruction of DPNH with acid, in both methods the same low reading is obtained in the absence of DPN^+. In contrast to the strong alkali method, N^1-methylnicotinamide gives a strongly fluorescent product with acetone or methyl ethyl ketone (7, 8).

Fluorescence from DPNH

Oxidation of DPNH—Levy and Hunter observed that if DPNH is treated with peroxide it then produces fluorescence in strong alkali.[5] When this was studied more closely, it was found that peroxide (room temperature) will not oxidize DPNH at pH 8 or pH 12 (no change in absorption at 340 mμ), but in 6 N NaOH it will produce a fluorescent product.[6] This is presumably accomplished by first oxidizing DPNH to DPN^+, since the fluorescence is quantitatively equal to that obtained from DPN^+ and behaves in every way the same. Full fluorescence requires somewhat longer to develop than with DPN^+, no doubt due to slowness of the oxidation.

Full fluorescence is obtained in an hour at 38° with 1 to 3 mM H_2O_2, whereas with higher H_2O_2 levels destruction occurs (Table III). DPNH and TPNH have been indistinguishable in their behavior toward H_2O_2 and in the fluorescence produced.

[5] Jerome F. Levy and F. Edmund Hunter, Jr., personal communication.

[6] Sung and Williams (9) added peroxide to trichloroacetic acid in preparing tissue extracts in the hope of oxidizing DPNH and TPNH and thereby preventing destruction in acid prior to analysis for total pyridine nucleotides. We have been unable to show any oxidation of DPNH either at room temperature or in ice water under the conditions prescribed by Sung and Williams (5 per cent H_2O_2 in 1.6 per cent trichloroacetic acid). Burton and Lamborg (10) found that DPNH could be oxidized in neutral or slightly alkaline solution if Cu or other metals were present. The oxidation, however, was accompanied by substantial destruction.

Destruction of DPN+—With alkali weaker than 0.01 N, destruction of DPN+ is excessively slow. With alkali stronger than 0.05 N, appreciable fluorescence develops during DPN+ destruction. Between these limits after treatment for 15 minutes at 60° or 2 hours at 38° the fluorogenicity is reduced to less than 1.5 per cent of the initial value. An hour in 0.02 N NaOH at 60° had no discernible effect on DPNH. Approximate half times for destruction of DPN+ have been found to be, respectively, 12, 5, and 3.5 minutes at 38° in 0.005, 0.02, and 0.04 N NaOH and 2 and 0.5 minutes at 60° in 0.005 and 0.02 N NaOH.

TABLE III

Effect of Peroxide on Fluorescence from DPNH and DPN+

The peroxide was allowed to act at 38° in 6.5 N NaOH at the concentrations indicated, and the samples were then diluted 10-fold to be read. The values are expressed as per cent of the fluorescence obtained with an equivalent amount of DPN+ in the absence of H_2O_2.

H_2O_2	30 min.			60 min.	
	DPNH	DPN+	TPN+	DPNH	DPN+
mM per l.					
0	21	97		26	100
0.5	67	98		89	99
1.1	81	97		96	98
3.3	94	95		97	96
10.2	94	94	91	93	93
29		84	80		
101		58	57		

A phosphate buffer may be substituted for dilute NaOH to permit more latitude in bringing well buffered solutions to the proper alkaline pH. Final ratios of $HPO_4^=$ to $PO_4^=$ from 2:1 to 1:3 were satisfactory. Carbonate buffers are not sufficiently alkaline.

The residual fluorescence obtained after destruction of DPN+ is the same after further treatment with 6 N NaOH alone or with 6 N NaOH containing 0.01 per cent H_2O_2.

Reproducibility and Interfering Substances—To obtain maximal reproducibility in measuring DPNH in the presence of a large excess of DPN+, it is necessary to be sure that the sample is well mixed during the destruction of DPN+ and that none of the sample escapes contact with the alkali. Anything which reacts with peroxide can interfere in sufficiently high concentration. Thus glucose or glucose-6-phosphate concentration must be kept below 1 mM during the alkaline reaction, or else the peroxide concentration must be increased.

Native DPNH Fluorescence

The simplest method for measuring DPN^+ or TPN^+ reduction is by the native fluorescence of the reduced nucleotides. The only disadvantage is the 10-fold lower sensitivity as compared to the indirect procedure. It was found, however, that Mg, under certain circumstances, can greatly enhance the fluorescence of TPNH, and this might cause serious error if not recognized.

If a TPNH solution containing Mg is added to 0.003 N NaOH, the fluorescence nearly triples (Fig. 4). Under the same circumstances, DPNH fluorescence is only increased by a third to a half. Very low concentra-

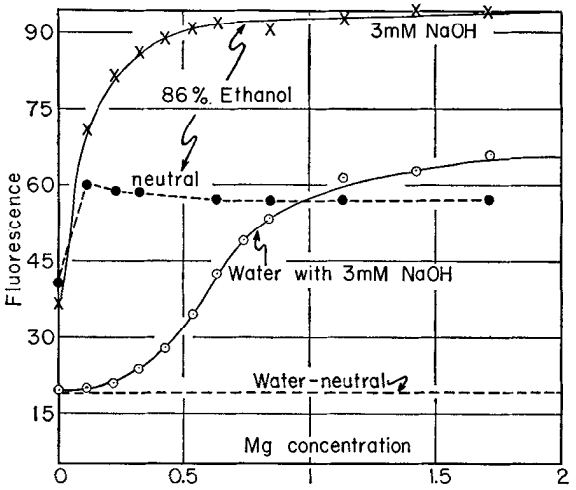

FIG. 4. Effect of Mg and alkali on fluorescence of TPNH in water and ethanol

tions of Mg are required (Fig. 4). The fluorescence is not constant. It reaches a peak in 5 or 10 minutes, then falls slowly for several hours. If Mg is added to the alkali before the TPNH, there is little enhancement at first, but finally the fluorescence rises to an intermediate value. The lack of initial enhancement is probably due to prior precipitation of $Mg(OH)_2$, which is only slowly available for complex formation with TPNH. The maximal enhancement is in 0.003 N NaOH. Presumably, with more alkali the Mg^{++} concentration is reduced so low as to be ineffective.

Sr and Ca have almost no effect in water solutions (Table IV), but in organic solvents a wide variety of polyvalent cations are capable of enhancing the fluorescence of both DPNH and TPNH (Table IV). Furthermore some of the effects are observed in neutral solution. It will be noted that the organic solvents themselves enhance the fluorescence of the reduced nucleotides.

Versene, citrate, or tartrate can abolish or diminish the enhanced fluorescence due to Mg in water or alcohol. More Versene is required to erase

TABLE IV

Effect of Various Cations on Native Fluorescence of DPNH and TPNH in Four Solvents

The values are calculated as per cent of the fluorescence in water. DPNH and TPNH were either 2.5 or 5×10^{-5} M, and both compounds had the same fluorescence in water on an equimolar basis. The NaOH and NH_4OH concentrations were 3 and 10 mM, respectively. The cation concentrations were all 1 mM.

Solvent	Coenzyme	No addition	NaOH	NH_4OH	Mg^{++}	Mg^{++} (NaOH)	Ca^{++} (NaOH)	Mg^{++} (NH_4OH)	Ca^{++} (NH_4OH)	Sr^{++} (NH_4OH)	Zn^{++} (NH_4OH)
H_2O.....	DPNH	100	102	102	101	185	106	102	102	102	112
"	TPNH	100	103	102	101	281	109	102	108	103	139
Ethanol.....	DPNH	167	154*	167	647	517*	468*	760	685	580	952
"	TPNH	92	119*	78	343	229*	247*	192	235	242	335
n-Propanol....	DPNH	297	164	247	482	461	422	820	514	471	591
"	TPNH	74	137	57	95	297	189	79	72	70	88
Methyl Cello-solve.....	DPNH	209	127	226	728	455	358	697	702	588	263
" " ..	TPNH	173	148	192	752	421	348	728	720	604	263

*These ethanol concentrations were 90 per cent; all the rest were 99 per cent.

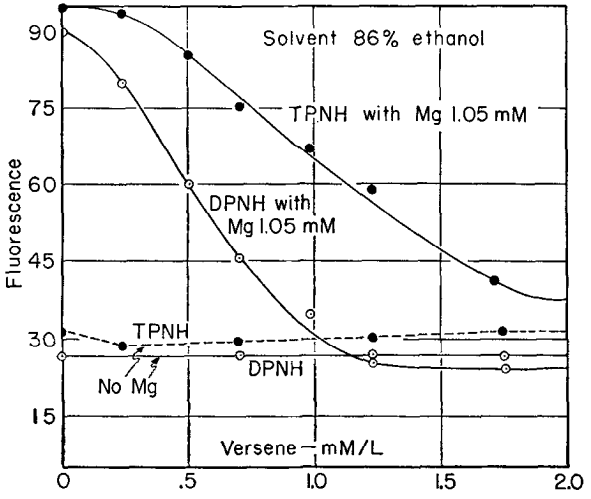

FIG. 5. Effect of Versene on fluorescence of DPNH and TPNH in 86 per cent ethanol in the presence of Mg.

the enhancement of TPNH fluorescence than of DPNH fluorescence (Fig. 5). This, together with the much smaller effect on DPNH than TPNH

in water, indicates that Mg is much more strongly bound by TPNH (with its extra phosphate group) than by DPNH in water or in 86 per cent ethanol. In *n*-propanol, under certain circumstances, TPNH is much less fluorescent than DPNH (Table IV). The absorption spectrum of TPNH is shifted slightly (3 to 5 mμ) to longer wave lengths by Mg in 0.003 N NaOH. The fluorescence spectrum is narrowed and shifted to shorter wave lengths both by Mg in dilute alkali and by organic solvents. The greatest shift occurs with Mg in an organic solvent (Fig. 3).

The enhancement of TPNH and DPNH fluorescence might be expected to serve some analytical purpose, but the disadvantages seem to outweigh most advantages. The enhanced fluorescence is not very stable. There is usually some precipitated hydroxide present, and the nucleotides appear to be absorbed in part on the precipitate; thus, fluorescence is affected by remixing. The greatest enhancement occurs in organic solvents. Unfortunately it is difficult to remove the last traces of fluorescence from organic solvents, consequently the optical blanks are much larger than in water. For this reason, when more sensitivity is needed, the conversion of DPNH to DPN^+ and thence to the strong alkali product is the preferred procedure.

Possibly some advantage might be taken of the differential effect of Mg on TPNH, but it seems preferable to distinguish between TPNH and DPNH with appropriate enzymes (see below). A more practical application may be in the measurement of minute amounts of divalent cations.

The temperature coefficient of DPNH fluorescence is −1.6 per cent degree between 20° and 38°. DPNH and TPNH in water or dilute alkali, in the absence of polyvalent cations, give within analytical limits the same fluorescence per mole. Also the fluorescent spectra (Fig. 3) are indistinguishable.

Illustrative Data

The results of assays for lactic dehydrogenase and for 6-phosphogluconate dehydrogenase in single large nerve cell bodies (Table V) can serve to illustrate the sensitivity and over-all reproducibility of measurement of DPN^+ in the presence of DPNH and the measurement of TPNH in the presence of TPN^+ (by the strong alkali method). It is expected that some variation should exist from one single cell to the next. The cell body capsules are particularly variable because of dissection difficulties. The values from the control area of Ammon's horn may be the best measure of reproducibility.

Glucose-6-phosphate dehydrogenase in brain has a very low K_m for TPN^+. By direct measurement of TPNH formation it was possible to work with very low TPN^+ levels and easily assess the Michaelis constant (Fig. 6).

Further illustration of the use of the procedures presented may be seen in methods for glutamic dehydrogenase, glutamic-aspartic transaminase in retinal samples of 0.1 γ (11), and single nerve cell bodies (12). A method

TABLE V

Lactic Dehydrogenase and 6-Phosphogluconic Dehydrogenase in Single Nerve Cell Bodies (Rabbit)

The activities are recorded as moles per kilo of dry weight per hour. The dry weight of each sample is given. Lactic dehydrogenase (LDH) was measured by the DPN^+ formed from pyruvate and DPNH. Incubations were in 2 μl. for 30 minutes. Fluorescence was read in 1 ml. 6-Phosphogluconic dehydrogenase (6PGDH) was measured by the TPNH formed from 6-phosphogluconate and TPN^+. Incubations were in 1 μl. for 60 minutes. Fluorescence was read in 60 μl. by the indirect method.

Dorsal root ganglion cell bodies				Anterior horn cell bodies			
Weight	LDH activity	Weight	6PGDH activity	Weight	LDH activity	Weight	6PGDH activity
mμgm.		mμgm.		mμgm.		mμgm.	
8.9	44.6	15.1	0.96	6.9	27.6	6.5	0.75
6.4	41.2	12.2	0.96	12.1	26.8	5.2	0.65
8.3	49.2	5.5	1.00	9.2	26.3	5.7	0.65
11.5	57.2	6.7	1.25	8.5	24.0	5.3	0.68
9.1	50.2	11.9	0.92	8.7	24.8	8.3	0.99
		13.1	1.12	7.9	29.5	7.7	0.68
		8.7	1.00	9.7	21.5	5.6	0.70
				5.3	27.7		
Average.....	48.5		1.03		26.0		0.73
Capsules of dorsal root ganglion cell bodies				Control region of dendrites*			
8.6	21.3	20.7	1.29	13.1	53.6	14.8	0.86
13.0	18.4	14.3	2.00	13.6	53.7	13.3	0.81
11.2	23.5	15.3	1.71	13.8	56.2	16.7	0.78
11.8	26.7	5.7	1.57	11.5	54.6	9.7	0.81
12.8	17.3	9.5	1.73	13.8	55.8	15.1	0.79
6.7	19.1			15.3	55.3	12.1	0.80
7.0	21.7			19.1	53.9		
Average.....	21.1		1.66		54.7		0.81

* Zona radiata from Ammon's horn.

for malic dehydrogenase based on the fluorometric determination of DPN^+ formed with oxalacetate is sufficiently sensitive to measure this enzyme in one large mitochondrion (12).

A final illustration of the use of these methods is in the measurement of the naturally occurring pyridine nucleotides in brain and liver (Table VI). The values were highly reproducible, and the sums of the separately meas-

ured nucleotides closely approximated the directly observed totals for oxidized PN, reduced PN, and total PN. However, judging from the results with a known mixture, the reduced nucleotide values may be 10 or 15 per

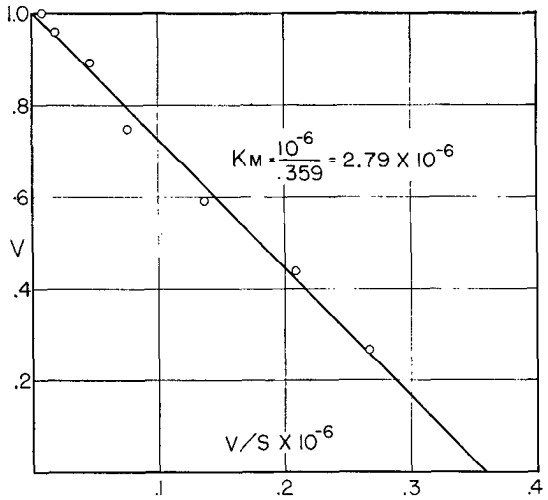

FIG. 6. Plot for assessing Michaelis constant of TPN^+ with rabbit brain glucose-6-phosphate dehydrogenase. The initial velocities were calculated from direct readings of the native fluorescence of TPNH as formed. Initial TPN^+ concentrations ranged from 10^{-4} to 10^{-6} M.

TABLE VI

Measurement of Native Pyridine Nucleotides in Liver and Brain

All measurements were made in triplicate. The pooled standard deviations were 0.005 and 0.0016 mM per kilo for liver and brain, respectively.

	Liver	Brain	Known mixture	
			Calculated	Observed
	mM per kg.	*mM per kg.*	*mM per l.*	*mM per l.*
DPN^+	0.542	0.241	0.306	0.330
TPN^+	0.071	0.010	0.107	0.109
DPNH	0.218	0.107	0.222	0.203
TPNH	0.063	0.012	0.114	0.097
$DPN^+ + TPN^+$, observed	0.588	0.245	0.413	0.417
" + " by summation	0.613	0.251	0.413	0.439
DPNH + TPNH, observed	0.280	0.116	0.336	0.288
" + " by summation	0.281	0.119	0.336	0.300
Total PN, observed	0.903	0.373	0.749	0.720
" " by summation	0.894	0.370	0.749	0.739

cent low, possibly due to oxidation and destruction during the initial heating step.

Evidence of specificity for total PN was obtained by measuring the rate of destruction of the alkali product with ultraviolet light. The rates were the same for those for known DPN^+ and for tissue extracts down to blank values.

Dr. Paul Greengard has developed procedures for measuring the tissue levels of a number of substrates through reduction or oxidation of pyridine nucleotides. The oxidized nucleotides are measured by the fluorescence developed with methyl ethyl ketone, the reduced nucleotides by their native fluorescence (13).

SUMMARY

1. The fluorometric method of Kaplan, Colowick, and Barnes has been adapted to permit the measurement of oxidized diphosphopyridine nucleotide (DPN^+) or triphosphopyridine nucleotide (TPN^+) at concentrations as small as 10^{-8} M in the presence of large excesses of reduced diphosphopyridine nucleotide (DPNH) or triphosphopyridine nucleotide (TPNH).

2. Similarly a method with the same sensitivity is presented for measuring DPNH or TPNH in the presence of large excesses of DPN^+ or TPN^+. The oxidized nucleotide is destroyed in dilute alkali, after which the reduced nucleotide is treated with peroxide in strong alkali. This converts it first to DPN^+ or TPN^+ and then to the fluorescent product of Kaplan *et al.* in one analytical step.

3. Alternatively the reduced nucleotides may be measured by their native fluorescence. Fluorescence of TPNH but not that of DPNH is enhanced 3-fold by Mg in dilute alkali. The fluorescence of both DPNH and TPNH is greater in organic solvents than in water and is further increased by a variety of cations.

4. Possible uses of the methods are illustrated by measurements of the lactic dehydrogenase and 6-phosphogluconate dehydrogenase activities of large single nerve cell bodies, by evaluation of the Michaelis constant for TPNH with brain glucose-6-phosphate dehydrogenase (2.8×10^{-6} M), and by determinations of individual values for DPN^+, DPNH, TPN^+, and TPNH in brain and liver.

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