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The Measurement of Pyridine Nucleotides by Enzymatic Cycling*

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In 1935 Negelein and Haas described a method for the determination of glucose 6-phosphate dehydrogenase activity based on measurement of the rate of reduced triphosphopyridine nucleotide formation from triphosphopyridine nucleotide (1). Since that time innumerable enzymes and substrates have been measured by the appearance or disappearance of reduced diphosphopyridine nucleotide or reduced triphosphopyridine nucleotide. In fact, with the aid of auxiliary enzymes nearly every substance of biological interest could be measured with a pyridine nucleotide system.

The present practical limit of sensitivity of spectrophotometric measurement of DPNH or TPNH is of the order of 10^{-5} moles per liter or 10^{-9} moles total.[1] The present practical limit for fluorometric measurement of reduced or oxidized pyridine nucleotides is of the order of 10^{-8} moles per liter or 10^{-12} moles total. Greater sensitivity would be useful both in respect to absolute amount measurable and in respect to concentration necessary for measurement.

This paper describes methods for determination of reduced or oxidized DPN or TPN at concentrations as low as 10^{-9} M and in amounts as small as 10^{-15} moles. It also indicates the possibility of extending the methods to the measurement of 10^{-19} moles of either pyridine nucleotide. The coenzyme to be determined is made to catalyze an enzymatic dismutation between two substrates. After several thousand cycles one of the products is measured.

Apparently, Warburg, Christian, and Griese were the first to use the cycling principle to measure TPN (3) in a system consisting of glucose-6-P and its dehydrogenase and "old yellow enzyme." In 10 minutes, for each mole of TPN, 330 moles of O_2 were consumed. Jandorf, Klemperer, and Hastings (4) used the cycling principle to measure DPN in a system containing enzymes to catalyze the conversion of fructose diphosphate to glycerol-P and P-glycerate. Each mole of DPN caused the release from bicarbonate buffer, in 1 hour, of 1300 moles of CO_2. With the use of this system in the Cartesian diver, Anfinsen (5) was able to measure with precision as little as 2×10^{-12} moles of DPN. More recently, Glock and McLean (6) measured pyridine nucleotides with cycling procedures. DPN was reduced with alcohol dehydrogenase, TPN with glucose-6-P dehydrogenase. They were then reoxidized by DPN or TPN cytochrome *c* dehydrogenase. Reduced cytochrome *c*, in 30- to 50-fold yield, was measured spectrophotometrically.

The system described here for TPN measurement, utilizes glucose-6-P dehydrogenase and glutamic dehydrogenase:

$$\text{TPNH} + \alpha\text{-ketoglutarate} + \text{NH}_4^+ \rightarrow \text{TPN}^+ + \text{glutamate} \quad (1)$$

$$\text{TPN}^+ + \text{glucose-6-P} \rightarrow \text{TPNH} + \text{6-P-gluconate} \quad (2)$$

Under the conditions given, each molecule of TPN catalyzes the formation of 5,000 to 10,000 molecules of 6-P-gluconate in 30 minutes. The 6-P-gluconate is then measured in a second incubation with 6-P-gluconate dehydrogenase and extra TPN^+. The TPNH produced is determined fluorometrically (7).

DPN is measured with lactic dehydrogenase and glutamic dehydrogenase:

$$\text{DPNH} + \alpha\text{-ketoglutarate} + \text{NH}_4^+ \rightarrow \text{DPN}^+ + \text{glutamate} \quad (3)$$

$$\text{DPN}^+ + \text{lactate} \rightarrow \text{DPNH} + \text{pyruvate} \quad (4)$$

Pyruvate is produced in 2500-fold yield in 30 minutes and is measured in a second step with added DPNH and lactic dehydrogenase. The resulting DPN^+ is measured fluorometrically (7).

During cycling, the nucleotides are used at concentrations well below their Michaelis constants; consequently, reaction rates are proportional to nucleotide concentrations. Since the final product in each case is again a pyridine nucleotide, the cyclic process can be repeated if necessary with an over-all multiplication factor of 10^6 to 10^8.

The method can be used to measure either the oxidized or the reduced form or the sum of both. In measuring DPN^+ (TPN^+), destruction of both DPNH (TPNH) and interfering enzymes, can usually be accomplished by brief acid treatment. In measuring DPNH (TPNH), mild alkaline treatment is ordinarily adequate for destruction of DPN^+ (TPN^+) and interfering enzymes. A companion paper (8) gives rate constants for destruction of oxidized and reduced pyridine nucleotides over a wide range of pH value and temperature.

EXPERIMENTAL PROCEDURE

Determination of TPN^+ or TPNH

Sample Procedure—The complete cycling mixture is made in 0.1 M Tris buffer, pH 8.0 (0.04 M Tris base-0.06 M Tris hydro-

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[1] This applies only to direct measurements of samples in a cuvette. Thus, 0.05 ml of a solution of DPNH with an optical density of 0.12 at 340 mμ contains 10^{-9} moles. By microscopic techniques, applied to living tissues or to suspended droplets (2) much smaller quantities, possibly as little as 10^{-14} moles, can be measured spectrophotometrically.

chloride) and contains 5 mM α-ketoglutarate, 1 mM glucose-6-P, 0.1 mM ADP, 0.025 M ammonium acetate, 0.2 mg per ml of bovine plasma albumin, 0.2 mg per ml of crystalline beef liver glutamic dehydrogenase, and sufficient yeast glucose-6-P dehydrogenase to give a calculated activity in the reagent at 25° of 0.6 mole per liter per hour with optimal TPN^+ and glucose-6-P concentrations. The required glucose-6-P dehydrogenase activity was provided by a concentration of 0.05 mg per ml of this enzyme as supplied (1960) by Boehringer and Sons, Mannheim, Germany. It may be assayed fluorometrically in 0.1 M Tris buffer, pH 8.0, with 0.1 mM TPN^+ and 1 mM glucose-6-P. The specified glutamic dehydrogenase concentration is sufficient to give a calculated velocity with optimal substrate and TPNH concentrations of approximately 0.4 mole per liter per hour at 25°.

The enzymes are added to the reagent within a few hours of use. If the enzymes are suspended in ammonium sulfate, the amounts to be used are centrifuged and resuspended in sufficient 2 M ammonium acetate to provide the necessary concentration of NH_4^+ indicated above. This reduces the sulfate concentration to 5 mM or less. (See "Kinetics of Enzymes Used for TPN Measurement.")

Volumes of 100 μl of complete cycling mixture (kept near 0°) are pipetted into serological tubes (7 × 75 mm) in a rack in ice. TPN^+ or TPNH (1 to 20 μl) is added to give concentrations in the range of 3×10^{-9} to 5×10^{-8} M. Water, or better a fluid closely approximating that containing the TPN, is added to bring all samples to the same volume ±2%. The rack is transferred to a 38° bath for 30 minutes and then to a 100° bath for 1½ to 2 minutes. From each tube 50 μl is transferred to a 3-ml fluorometer tube containing 1 ml of 0.02 M Tris buffer, pH 8, with 0.02 mM TPN^+, 0.1 mM EDTA, and sufficient 6-P-gluconate dehydrogenase to oxidize 50% of 0.005 mM 6-P-gluconate in 3 minutes or less. After 30 minutes at room temperature, the fluorescence of each sample is measured, together with that of control samples containing 6-P-gluconate in the range anticipated (7,000- to 10,000-fold yield). Standards which increase in steps of two or three to cover the particular concentration range are provided. Standards and blanks are carried through the entire process, including any procedure before cycling.

The final 6-P-gluconate concentration in the fluorometer should not exceed 10^{-5} M since this is the limit of proportionality of TPNH fluorescence. Higher levels could be measured spectrophotometrically, but there is no obvious advantage.

The 6-P-gluconate dehydrogenase used here, was prepared from rat liver (see "Special Preparations"). It is stable for at least 1 hour at room temperature in the final reagent.

The cycling reagent without enzymes may be stored for 2 weeks at −20° or 2 months at −85°. Longer storage results in loss of α-ketoglutarate.

Permissible Variations—The cycling procedure is quite flexible. The *time* of incubation may be varied between 15 and 60 minutes to accommodate higher or lower TPN concentrations. An hour's incubation is recommended for samples in the range of 1 to 3×10^{-9} M TPN, and an aliquot of 90 to 100 μl may be used for the last step to increase sensitivity. The cycling rate is linear for 30 minutes, but falls off a little between 30 and 60 minutes (Fig. 1).

The *volume* during cycling may be increased, but the cost of the enzymes may be a deterrent. However, if there is a prejudice against the use of small volumes, and 2000- to 3000-fold

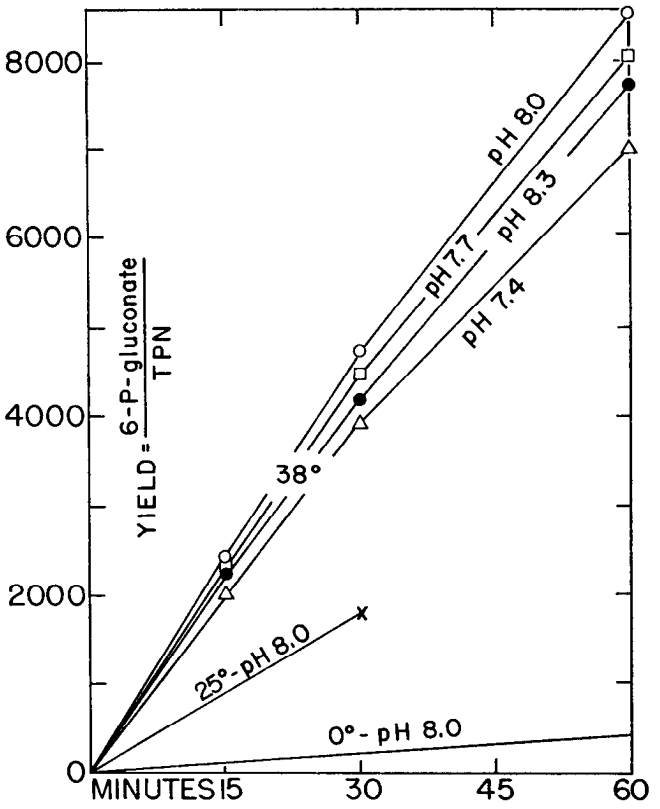

FIG. 1. Effect of pH value, temperature, and time on TPN cycling. Incubation volume was 100 μl. The TPN concentrations were 2.9×10^{-8} M for samples at 25 and 38°, and 2.9×10^{-7} M for samples at 0°. The enzyme concentrations were 75% of those described in the sample procedure in text.

cycling is sufficient, the enzyme concentrations may be reduced to 0.2 of those given above and larger cycling volumes employed. In this case ammonium acetate is added to keep the concentration of NH_4^+ at approximately 0.025 M. The cycling rate is not reduced in direct proportion to enzyme concentration as discussed below. Conversely, the cycling volume may be reduced, if necessary, to as little as 1 μl in appropriately smaller tubes with corresponding increase in sensitivity (Table I). If the absolute amount of 6-P-gluconate formed is less than 5×10^{-10} moles, it is usually desirable to measure indirectly the final TPNH produced (see "Indirect Measurement of TPNH").

The *temperature coefficient* is 6.4% per degree between 0 and 25° and 7.7% per degree between 25 and 38° (Fig. 1). The rate at 0° is 8% of that at 38°. Therefore, the time between addition of first and last sample to the cycling mixture should not exceed half the subsequent incubation time if the limit of tolerance is 4%.

The *optimal pH* is 8.0, but the rate is only 7% lower at pH 7.7, and 11% lower at pH 8.3 (Fig. 1).

The *glucose-6-P* concentration may be increased to 5 mM to permit TPN concentrations up to 3×10^{-7} M. The only disadvantage is a slight increase in cycling blank. This substrate can conversely be reduced to 0.3 mM with very low TPN levels, with only slight sacrifice of cycling efficiency, since the Michaelis constant is approximately 0.02 mM in the cycling mixture. The *α-ketoglutarate* concentration cannot be substantially altered from that recommended without decreased cycling. It does not contribute an appreciable blank. The *ADP* level is not

TABLE I

Cycling in small volume and double cycling

Two sets of samples were cycled for 30 minutes at 38°. The TPN *concentrations* were the same in both sets (5 and 20 × 10^{-9} M) but the volumes during cycling differed by a factor of 100. The 100-μl samples were subsequently treated according to the "sample procedure" given in the text. The 1-μl samples, after cycling, were analyzed for 6-P-gluconate as described under "Indirect Measurement of TPNH." The procedure followed for the double cycling experiment is given in the text. For double cycling, the first step was carried out in 1 μl, the second cycling step in 100 μl with 4.7% of the original sample (see text).

Single cycling (100 μl)			Single cycling (1 μl)			Double cycling		
TPN	6-P-Glucose	Yield*	TPN	6-P-Glucose	Yield*	TPN	6-P-Glucose	Yield*
10^{-13} *mole*	10^{-9} *mole*		10^{-15} *mole*	10^{-11} *mole*		10^{-16}† *mole*	10^{-8} *mole*	
5.55	4.1	7420	5.6	4.4	7900	2.6	1.20	4.6 × 10^{7}
5.55	4.3	7760	5.6	3.8	6800	2.6	1.13	4.3 × 10^{7}
5.55	4.3	7760	5.6	3.8	6900	2.6	1.27	4.9 × 10^{7}
5.55	4.3	7760	5.6	3.6	6400	2.6	1.24	4.8 × 10^{7}
20.5	14.5	7050	20.7	13.6	6600	9.7	3.90	4.0 × 10^{7}
20.5	14.8	7230	20.7	14.2	6900	9.7	3.77	3.9 × 10^{7}
20.5	15.0	7330	20.7	14.8	7200	9.7	3.88	4.0 × 10^{7}
20.5	14.8	7220	20.7	14.9	7200	9.7	3.83	4.0 × 10^{7}

* Ratio of 6-P-gluconate produced to TPN originally present.

† Calculated from the fraction carried through both cycling steps.

critical; in fact, the cycling rate is only 20% lower when it is omitted (30-minute cycling). However, it appears to protect glutamic dehydrogenase, particularly for longer periods of incubation. The NH_4^+ level is not critical, but there is some inhibition if the concentration is increased to 0.1 M (due to increase of apparent K_m for TPN^+ with glucose-6-P dehydrogenase).

Source of Blanks—Both cycling procedures are capable of giving reproducible results, with coenzyme concentrations during cycling as low as 1 or 2 × 10^{-9} M. At these levels the blank values become critical. In the sample procedure above, the over-all blank value need not exceed the equivalent of 3 × 10^{-9} M TPN (calculated as concentration during cycling). This blank has three sources: (*a*) The cycling reagent contains materials which fluoresce slightly at pH 8. These may account for a third or more of the blank. (*b*) The final 6-P-gluconate dehydrogenase reagent may have a fluorescence blank equal to that of 3 × 10^{-7} M TPNH measured directly. This would be equivalent to approximately 10^{-9} M TPN concentration during cycling. A third of this blank appears to be due to water itself, a third is due to the Tris buffer, and a third is due to TPN^+ and the dehydrogenase. Higher readings indicate dirty tubes or contaminated solutions. It is recommended that all tubes be cleaned by heating 15 minutes at 100°, first in half-concentrated HNO_3, then in distilled water, with a rinse in redistilled water. (*c*) There may be a small increase in blank resulting from incubation of the complete cycling reagent. This presumably indicates the presence of minute amounts of TPN. This need not exceed the equivalent of 5 × 10^{-10} M TPN. The first two contributions to the blank may be, in effect, reduced 10-fold by measuring TPNH indirectly (see below).

A penalty attached to the high sensitivity of the cycling methods is the danger of contamination with coenzymes themselves. Pipettes that are to be used at any point where contamination could interfere should not be used to pipette strong coenzyme solutions, or else they should be specially cleaned by soaking for 1 hour (inside and out) in 0.1 N NaOH, and rinsed with 0.1 N HCl. It would seem wise to keep stock coenzyme solutions away from the cycling reagents.

Indirect Measurement of TPNH—With smaller samples the sensitivity may be increased 10-fold by measuring the final TPNH indirectly as follows. After cycling and heating to 100°, each sample is treated for 30 minutes with 3 to 10 volumes of 6-P-gluconate dehydrogenase reagent. A solution of 0.3 M Na_3PO_4-0.3 M K_2HPO_4 is added in an amount equal to twice the volume used for cycling and the sample is heated 10 minutes at 60° to destroy excess TPN^+. An aliquot is then added to 0.2 ml of 6 N NaOH containing 0.03% H_2O_2 (8) in a fluorometer tube. After heating for 10 minutes at 60°, 1 ml of water is added, and the fluorescence measured.

Kinetics of Enzymes Used for TPN Measurement—(The kinetics of rat liver *6-P-gluconate dehydrogenase* are discussed below where the preparation is described.) During cycling, pyridine nucleotide concentrations are far below the Michaelis constants. Therefore, the important kinetic factor is the first order rate constant $k = V_{max}/K_m$, where V_{max} is the velocity with saturating levels of coenzyme. With establishment of steady state, the rate of TPN^+ reduction must equal the rate of TPNH oxidation, *i.e.* $k_a(TPN^+) = k_b(TPNH)$. The over-all cycling rate constant, k_c, is equal to $k_a k_b/(k_a + k_b)$.

Since $k_a/k_b = (TPNH)/(TPN^+)$, the ratio of the two rate constants can be observed directly in the fluorometer with cycling mixture to which is added a low but measurable concentration of TPN^+ (10^{-6} M). With a moderate concentration of glucose-6-P, *e.g.* 5 × 10^{-4} M, the cycling process can be followed directly, since the TPNH concentration will first hold nearly constant at a steady state level and then fall sharply when the substrate is nearly used up. This procedure was used to determine optimal cycling conditions. It could also be used as a quick test of the adequacy of the complete cycling reagent if difficulty should arise.

Yeast glucose-6-P dehydrogenase in 0.1 M Tris, pH 8, has a K_m value for glucose-6-P of 0.02 mM, and for TPN^+ of approximately 0.002 mM. There is very little mutual dependence of

the K_m value for either TPN^+ or glucose-6-P on the concentration of the other. The V_{max} is not affected by α-ketoglutarate, NH_4^+, or sulfate in the amounts present in the cycling reagent, but they all depress the first order rate constant for TPN^+. There is approximately 30% inhibition by 5 mM α-ketoglutarate or 0.1 M ammonium acetate. Sulfate is particularly inhibitory and the inhibition varies with the square of the concentration. With 1 mM glucose-6-P, and 0.002 mM TPN^+, sulfate at concentrations of 0.005, 0.010, 0.018, and 0.036 M, inhibited 40, 55, 88, and 98%, respectively. It is therefore important to remove most of the sulfate from the enzyme preparations before use. There is no appreciable inhibition of the first order rate constant by the products, glutamate and 6-P-gluconate, at 0.5 mM concentration. The net effect of the various inhibitors in the cycling reagent is to give an apparent K_m value for TPN^+ of approximately 0.005 mM. With the amount of glucose-6-P dehydrogenase indicated in the sample procedure (V_{max} = 600 mmoles per liter per hour), the calculated first order rate constant for TPN^+ would approximate 120,000 per hour at 25°.

Glutamic dehydrogenase (beef liver) according to Frieden (9) has a K_m value of 0.7 mM for α-ketoglutarate and of 0.026 mM for TPNH at pH 8.0 in 0.01 M Tris-acetate at 25°. In the present cycling mixture at 25° the K_m value for TPNH was found to be approximately 0.01 mM with a V_{max} of 400 mmoles per liter per hour for a 0.2 mg per ml solution. This would give a calculated first order rate constant of approximately 40,000 at 25°. (The constant is only a little higher at 38°.) Frieden observed (10) that ADP enhances the activity of glutamic dehydrogenase, particularly in the presence of high levels of DPNH and at all levels of TPNH tested. It has been found that with lower levels of TPNH than he used there is almost no effect of ADP on rate (pH 7.6 to 8.0). Lowering the TPNH (to 0.002 mM) has two other kinetic effects: The apparent Michaelis constant for NH_4^+ is lowered by a factor of at least 10 (to less than 0.5 mM) and the inhibitory effect of concentrated α-ketoglutarate (50 mM) is reduced to 40% (instead of 80% inhibition with 0.04 mM TPNH). At a more alkaline pH value (9.2) ADP has an accelerating effect even with low TPNH. Neither glutamate (0.5 mM) nor 6-P-gluconate (0.5 mM) were inhibitory to glutamic dehydrogenase activity in the cycling mixture (low TPNH levels).

Cycling rates observed directly in the fluorometer are approximately as predicted from the above kinetic considerations when the level of enzymes is kept relatively low. With the high enzyme levels of the cycling mixture above, the rates are only approximately $\frac{1}{3}$ of those predicted. Observation of steady state levels of TPNH in this case indicates that the rate of TPN^+ reduction is much less than expected. It seems probable that a substantial portion of the TPN^+ is combined with the two enzymes, which are present in much greater equivalent concentrations than the nucleotide. Some of the TPNH is also bound by the enzymes as demonstrated by enhancement of TPNH fluorescence (in the absence of substrates).

Double Cycling

It is not the purpose of this paper to exploit the possibilities of double cycling. However, a single experiment may be described to illustrate the very high amplification attainable (Table I).

Samples containing 5 and 20 $\times 10^{-5}$ moles of TPN^+ were cycled at 1 μl volume for 30 minutes. After heating at 100°, TPN^+ and 6-P-gluconate dehydrogenase were added to make a total volume of 8 μl. This was incubated 15 minutes at 26°, after which surplus TPN^+ was destroyed by heating for 10 minutes at 60° with 2 μl of 0.3 M Na_3PO_4-0.3 M K_2HPO_4. Some of the samples were analyzed at this stage by heating with strong NaOH containing H_2O_2. A 7000-fold yield of TPNH had been obtained (Table I). Aliquots of the remaining samples were recycled at a volume of 100 μl. The aliquots used (0.5 μl) represented only 5% of each original sample. The final yield of TPNH was equal to a gain of approximately 45,000,000-fold. A surprising thing is that reproducibility did not suffer in spite of the high gain. As discussed below, to capitalize fully on double cycling it would be necessary to conduct the first stage in a very small volume.

Determination of DPN^+ or DPNH

Sample Procedure—The complete cycling mixture is made in 0.2 M Tris buffer, pH 8.4 (2:1 ratio of Tris base to Tris HCl), and contains 100 mM sodium lactate, 0.3 mM ADP, 5 mM α-ketoglutarate, 0.05 to 0.15 M NH_4^+, 0.4 mg per ml of crystalline beef liver glutamic dehydrogenase, and 0.05 mg per ml of crystalline beef heart lactic dehydrogenase (charcoal-treated see "Special Preparations"). The enzymes to be used are added within an hour of use. If they are provided as suspensions in strong $(NH_4)_2SO_4$ this can furnish the required NH_4^+. The amounts of enzymes used are such as to give calculated rates in the cycling mixture at 25°, with optimal levels of substrates and coenzymes, approximately of 1 mole per liter per hour for glutamic dehydrogenase and 0.12 mole per liter per hour for lactic dehydrogenase.

Volumes of 100 μl of complete cycling mixture (kept near 0°) are placed in 7 × 75 mm tubes in a rack in ice. DPN^+ or DPNH (1 to 20 μl) is added to give a concentration in the range 3 $\times 10^{-9}$ to 5 $\times 10^{-8}$. Water or a fluid approximating that containing the nucleotide is added to bring all samples to the same volume ±2%. The time between addition of first and last sample in a set should not exceed 10 minutes since the cycling rate is 15 to 20% as fast at 0° as at 25° (Fig. 2). The rack is transferred to a bath at 25° for 30 minutes and then to a bath at 100° for $1\frac{1}{2}$ to 2 minutes.

To each tube in ice is added 100 μl of a reagent containing 0.65 M NaH_2PO_4, 0.15 M K_2HPO_4, 1.5 μg per ml of crystalline rabbit skeletal muscle lactic dehydrogenase, and DPNH at a concentration 3 to 10 times that of the expected pyruvate (2500 times the DPN concentration). The lactic dehydrogenase is added to the ice-cold phosphate reagent within an hour of use, and the DPNH within 15 minutes of use. The rack of samples is transferred to a water bath at 20–30° for 15 minutes and returned to the ice bath. To each sample is added at once 25 μl of 5 N HCl with very thorough mixing to destroy excess DPNH.

An aliquot of 100 μl is added to 200 μl of 9 N NaOH. After heating 10 minutes at 60°, 1 ml of H_2O is added and the fluorescence read.

Since pyruvate formation is not strictly linear with DPN concentration, standards of DPN^+ are included which increase in steps of two to cover the range of assay. Blanks and standards are carried through the entire procedure, and are treated as nearly as possible like the samples to be analyzed.

Permissible Variations—The cycling procedure is not as flexible as that for TPN due primarily to a fall off in rate as pyruvate accumulates. Arbitrarily, a 0.1 mM pyruvate concentra-

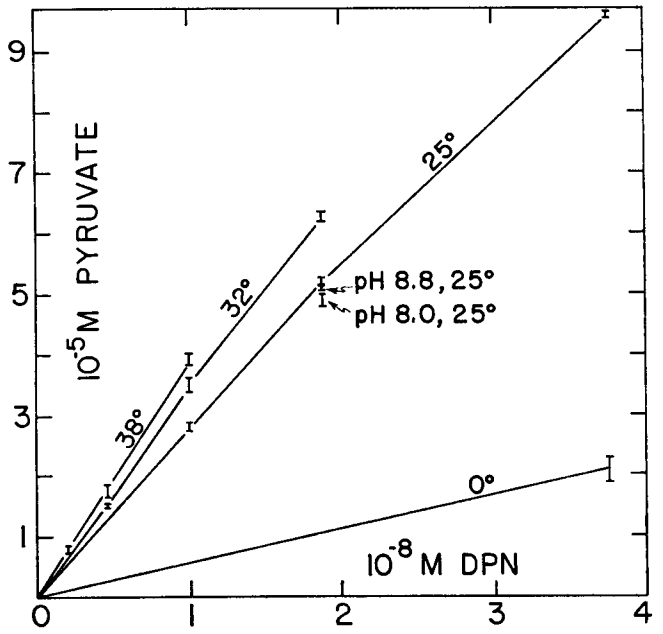

FIG. 2. Effect of temperature, pH value, and concentration of DPN on cycling. Incubation time was 30 minutes in a volume of 100 μl. The pH value was 8.4 unless otherwise indicated. The pyruvate concentration produced has been plotted against the concentration of DPN present. The range for quadruplicate determinations around each point is indicated.

tion, at the end of cycling, has been set as a practical upper limit. If this consideration is kept in mind, the incubation time may be extended to 60 minutes and probably longer to measure DPN levels in the 1 to 3 × 10^{-9} M range. Incubation for less than 30 minutes is not recommended, due to the substantial cycling rate at 0°, which would make it difficult to handle large numbers of samples at a time. For this reason it may be preferable in general to reduce lactic dehydrogenase to half and cycle for 60 minutes.

Cycling *volumes* may be increased without undue cost, since neither enzyme is very expensive. The cycling volume may also be decreased as in the case of TPN. No difficulty has been encountered with volumes of 5 μl and 10^{-8} M DPN.

When it is unnecessary to work with lowest DPN concentrations it may be desirable to decrease the *cycling rate*. This permits the use of higher DPN levels without excessive departure from linearity, and reduces the danger of variability from contamination. When the cycling rate is to be reduced it is recommended that lactic dehydrogenase be lowered more than glutamic dehydrogenase to keep the DPN^+ to DPNH ratio high and thereby minimize reduction of pyruvate formed. A reagent containing 50 mM lactate, 0.2 mg per ml of glutamic dehydrogenase, and 0.01 mg per ml of lactic dehydrogenase gave a yield of 600 moles of pyruvate per mole of DPN in 30 minutes.

The *temperature coefficient* is 7% per degree between 0 and 25°, but only 3% and 2.2% per degree between 25 and 32° and between 32 and 38°, respectively (Fig. 2). There is, therefore, only a modest gain in raising the temperature above 25°. However, there is no reason why the cycling cannot be carried out satisfactorily at 38°, since the complete reagent is quite stable at this temperature.

The optimal cycling *pH* value is 8.4, but it is only diminished 2% and 7% by changing the pH to 8.8 and 8.0, respectively (Fig. 2).

The *lactate* concentration is kept high to minimize the inhibitory effects of pyruvate accumulation. Since the best commercial lactate contains at least 1 part in 40,000 pyruvate, there is some advantage in regard to blank in reducing lactate when measuring very low DPN levels (1 to 5 × 10^{-9} M). The initial cycling rate is as high with 50 as with 100 mM lactate. Substantial change in *α-ketoglutarate* concentration will decrease glutamic dehydrogenase activity but will not have much effect on cycling rate since lactate reduction is the rate-limiting step. The concentration of NH_4^+ is also less critical than for the TPN method.

Source of Blanks—A major contribution to the blank comes from pyruvate in the lactate. With 100 mM lactate as substrate, 1 part of pyruvate in 40,000 would equal 2.5 × 10^{-6} M or the equivalent of 10^{-9} M DPN (with 2500-fold cycling). The heart lactic dehydrogenase used (after charcoal treatment) contributed the equivalent of 2 × 10^{-10} M DPN. Both these blanks could probably be reduced if necessary. The remainder of the cycling components do not add appreciably to the blank. The DPNH used after cycling for pyruvate measurement adds a blank equal to 1 or 2% of the amount present. However, if the DPNH has become oxidized, it may contribute a much greater blank. The fluorescence from the water and alkali used for the last step ought not to exceed that from 1 or 2 × 10^{-8} M DPN^+ (direct reading), *i.e.* the equivalent of 1 or 2 × 10^{-10} M DPN as cycled in the sample procedure. The over-all blanks tend to be more erratic for DPN than for TPN. This is attributed, on the basis of considerable evidence, to contamination by DPN itself. Apparently, DPN is more abundant than TPN in the laboratory as well as in most tissues. Suggestions for avoidance of contamination have been given in the TPN section.

Kinetics of Enzymes Used for DPN Determination—Beef heart lactic dehydrogenase kinetics is rather complex. Under the recommended cycling conditions with 0.1 M lactate, the apparent K_m value for DPN^+ is 10^{-5} M and there is a nearly linear relationship between $1/v$ and $1/(DPN^+)$ with DPN^+ from 3 × 10^{-4} to 5 × 10^{-7} M. This indicates a first order rate constant for DPN^+ of approximately 12,000 per hour with 0.05 mg per ml of beef heart lactic dehydrogenase as given in the sample procedure. With low DPN^+ (2 × 10^{-6} M) velocity is half-maximal with 8.6 × 10^{-3} lactate. The kinetics of this enzyme has been very thoroughly studied by Schwert *et al.* (see for example Winer and Schwert (11)). The above K_m value for DPN^+ is only $\frac{1}{7}$ of the value they found under conditions that were quite similar except that these authors used semicarbazide to trap the pyruvate formed.

Glutamic dehydrogenase at a concentration of 0.4 mg per ml and pH 8 to 8.4 gives a calculated first order rate constant for DPNH of 60,000 per hour (25°). Glutamic dehydrogenase is deliberately used at this relatively high level of activity in order to maintain a high steady state ratio of DPN^+:DPNH for the reason already stated.

As with TPNH, there is only slight enhancement of glutamic dehydrogenase activity by ADP at pH 8 with low DPNH concentrations (0.002 mM). As the pH value is made more alkaline the velocity decreases rapidly and the ADP enhancement becomes more marked.

Lactate Pyruvate Equilibrium and Pyruvate Measurement—The

equilibrium constant for (pyruvate) (DPNH)/(lactate) (DPN^+) was determined to be 8.8×10^{-4} at pH 8.4 in 0.1 M Tris buffer. This gives an equilibrium constant of 3.9×10^{-12} which is in substantial agreement with values in the literature. Lactate is used at very high concentration during cycling to compensate for the unfavorable equilibrium. When it comes to pyruvate measurement the high level of lactate is a disadvantage and makes it necessary to lower the pH value as much as possible. At the pH value chosen, 6.5, (pyruvate) (DPNH)/(lactate) (DPN^+) = 1×10^{-5}. Any pyruvate remaining represents a negative error. This error, as a fraction of the total initial pyruvate, is equal to the final equilibrium value for (pyruvate)/(DPN^+). With a lactate concentration of 0.05 M after dilution in the second step, the equilibrium constant for (pyruvate)/(DPN^+) is equal to 5×10^{-7}/(DPNH).[2] This means that to prevent a negative error of more than 5%, the excess DPNH cannot be less than 10^{-5} M, or 0.02% of the lactate concentration. Direct observations confirm this calculation. To provide sufficient DPNH only becomes a problem when measuring lowest DPN levels. For example, a DPN concentration of 10^{-9} M with 2000-fold cycling results in 10^{-6} M pyruvate at the pH 6.5 step, and would require a 10-fold excess of DPNH.

For convenience, the DPNH and lactic dehydrogenase are added to the phosphate solution that is used to shift the pH value to 6.5. This solution because of its acidity (pH 6.2) and high phosphate concentration destroys DPNH at a rate of 40% per hour at 25° but only 5% per hour at 0°. Possible loss of DPNH is borne in mind in calculating the amount of DPNH to use.

ANCILLARY PROCEDURES

Preparation of Sample

The sensitivity of the cycling methods simplifies sample preparation. In the analysis of tissues no extraction is necessary before cycling because of the high dilution. Nor, does extraction seem desirable, since there is always a possibility that coenzyme might be adsorbed by insoluble material.

Ordinarily, before cycling, samples are treated with acid or alkali to destroy reduced or oxidized coenzymes, as the case demands, and this treatment can also accomplish the destruction of interfering enzymes that may be present. In general, it is sufficient for destruction of DPNH and TPNH to add HCl to give an acid excess of 0.02 to 0.05 N. Destruction is complete in 1 or 2 minutes at room temperature, whereas DPN^+ and TPN^+ are stable for several hours (or as long as a week at 4° in 0.02 N HCl, although freezing in acid results in loss). Similarly, heating for 10 minutes at 60° with a 0.02 N excess of NaOH destroys 99.99% of oxidized pyridine nucleotides without loss of reduced forms, and the weakly alkaline solution may be stored for several days at 4° (but not frozen) without appreciable loss (8).

Two situations have been encountered in which the acid or base pretreatment is more exacting. The first situation results from the extraordinary stability of mammalian DPNase toward acid. Therefore, in preparing tissues for measurement of native DPN^+ or TPN^+ it is recommended that they be homogenized at a dilution of at least 1:50 in 0.01 M H_2SO_4-0.1 M Na_2SO_4 with subsequent heating for 45 minutes at 60°. This reduces DPNase to a point at which it will ordinarily not be disturbing although some activity may persist (8).

The second situation demanding special treatment arises from the fact that blood in the presence of alkali can oxidize DPNH or TPNH. As little as 1 part of blood in 40,000 parts of 0.02 N NaOH will oxidize a substantial fraction of DPNH or TPNH in an hour at 25° or in 10 minutes at 60° (8). Blood itself may be treated at a dilution of at least 1:100 for 10 minutes at 60° in 0.1 N Na_2CO_3-0.02 N $NaHCO_3$ to destroy oxidized nucleotides with little loss of the reduced forms. However, this treatment is not satisfactory for all tissues. For example, rat liver homogenate at this pH value (10.7) quickly oxidizes some of the DPNH. A satisfactory solution appears to be to homogenize tissues at a dilution of at least 1:100 in ice-cold 0.02 N NaOH containing 0.5 mM cysteine, and to heat the homogenates (within an hour) for 10 minutes at 60° (8). The cysteine (as cysteine hydrochloride) is added to the NaOH solution shortly before use.

In preparing tissues for substrate analysis with a pyridine nucleotide system it will often be possible to destroy interfering enzymes ahead of time by treatment with acid or base. In this case, the two difficulties just discussed may not arise. If the substrate can tolerate heating with base (*e.g.* 5 minutes at 60° in 0.02 N NaOH), this is the treatment of choice since base is much more effective than acid in destroying DPNase. In measuring very low substrate levels, it may be necessary to make a special point of destroying native coenzymes before assay. For example, if glucose-6-P were to be measured with glucose-6-P dehydrogenase and TPN^+, the native tissue TPNH would need to be first destroyed with acid unless the TPNH concentration was insignificant in comparison to the glucose-6-P level. (Acid pretreatment in this case would suffice for both TPNH and enzyme destruction.)

When samples are ready for the cycling step in the assay, they may be handled in the same manner whether the coenzyme to be measured is the pre-existing native substance or the product of an enzyme reaction designed to measure an enzyme or substrate. Usually the required volume of the sample will be so small that whether it is acid or alkaline it can be added directly to the complete cycling mixture without prior neutralization. If the sample adds no more than 0.01 mole per liter of acid or base to the cycling mixture it will not change the pH value significantly, nor destroy more than a few per cent of the cycling enzymes. If this limitation is to be exceeded, the samples may be brought to the approximate pH value of the cycling mixtures by adding 2 moles of Tris base per mole of acid or 2 moles of Tris hydrochloride per mole of base, as the case may be. The Tris hydrochloride should have a pH value no lower than 6 to avoid danger of TPNH or DPNH destruction during addition. Storage is not recommended after neutralization.

If the volume of original sample to be used exceeds 20% of that of the cycling reagent, the reagent may be prepared in more concentrated form.

Special Preparations

6-P-Gluconate Dehydrogenase—Available commercial preparations of 6-P-gluconate dehydrogenase from yeast are contaminated with glucose-6-P dehydrogenase and are therefore unsuitable for the present purpose. Rat liver is a rich source (13) and was found to be at least twice as active as sheep, beef, or hog

[2] At pH 6.5, from the preceding equation, (pyruvate)/(DPN^+) = 10^{-5} (lactate)/(DPNH) = 5×10^{-7}/(DPNH).

liver. Fresh livers, 50 g to 200 g, were homogenized in 9 volumes of 0.025 M phosphate buffer at pH 7.5. (Throughout the preparation was kept at 0–4°. All centrifugations were made at approximately 10,000 × *g* for 20 to 30 minutes.) The initial activity was 21 mmoles per liter per hour measured in the fluorometer at 25° with 0.5 mM 6-P-gluconate, 0.05 mM TPN^+, and 1 mM EDTA in 0.05 M Tris at pH 8. The precipitates were discarded after centrifuging the original homogenate and after adding solid ammonium sulfate to concentrations of first 1 M and then 2 M. The precipitate obtained at an ammonium sulfate concentration of 3 M contained 70% of the original activity. This measured approximately 0.3 ml per g of liver. It was dissolved in 3 volumes of phosphate buffer (0.025 M, pH 7.5) and dialyzed 5 hours at 4° against this same buffer containing 0.2 mM EDTA. The solution was diluted to a protein concentration of 1% (measured with Folin phenol reagent) and nucleic acid was removed with 0.04 volume of 1% protamine sulfate. After centrifuging, solid ammonium sulfate was added to the supernatant fluid to a concentration of 2 M with enough 1 N NH_4OH to bring the pH value to 7. The precipitate was discarded and the activity (55% of the original) was precipitated at 2.8 M ammonium sulfate concentration (neutralized). After dialyzing as before, the sample was brought to a protein concentration of 1% and adsorbed on $Ca_3(PO_4)_2$ gel. The gel was added in three steps (1.5 ml of 0.4% gel per ml of sample at each step). The gel was removed by centrifuging after each addition. The third gel treatment adsorbed half the activity remaining at that stage, and 60% of this activity was recovered by eluting with 0.2 M phosphate buffer, pH 7.4, (40 ml per g of gel) and precipitation with 3 volumes of 4 M ammonium sulfate (neutralized). The activity was 30 moles per kg of protein per hour, assayed as above. The yield was approximately 12% with a 25-fold purification. Further ammonium sulfate fractionation resulted in loss of activity without gain in specific activity. Glucose-6-P dehydrogenase and hexokinase activity were 1 part in 10,000 and 1 part in 900, respectively, of the 6-P-gluconate dehydrogenase activity. The preparation also contained isocitrate dehydrogenase and a trace of malic enzyme. In 0.02 M Tris buffer at pH 8 the Michaelis constant for 6-P-gluconate is approximately 10^{-5} M, and that for TPN^+ about 3×10^{-7} M.

Purification of Heart Lactic Dehydrogenase—Beef heart lactic dehydrogenase (8 ml of a 2% suspension in 2.5 M ammonium sulfate (Worthington Biochemical Corporation)) was diluted to 40 ml with 2% Norit. The enzyme was then recrystallized from the supernatant fluid according to Neilands (12). This treatment reduced DPN^+ concentration from 8×10^{-4}, to 1×10^{-5} moles per kg of protein. Recrystallization without charcoal treatment did not remove appreciable DPN^+.

Preparation of Sodium Lactate—Five grams of calcium (L(+)-lactate)$_2 \cdot 4\ H_2O$ (California Corporation for Biochemical Research) were suspended in 32 ml of H_2O. After adding 11 ml of 2 M Na_2CO_3 the suspension was shaken vigorously and filtered. The alkaline filtrate was brought to pH 7 with approximately 1 ml of 5 N HCl. The solution assayed 0.78 M with acetyl-DPN and lactic dehydrogenase.

Table II

Reproducibility of TPN and DPN measurement

Cycling was carried out for the times indicated in a volume of 100 µl at 38° for TPN and 25° for DPN. The somewhat lower cycling rate for the three lowest TPN concentrations is attributed to the use of a 0.3 mM concentration of glucose-6-P (to diminish the blank) rather than 2 mM used for the other samples. To keep the final TPNH concentration within the range of the fluorometer, the aliquots taken finally into 1 ml for 6-P-gluconate determination varied from ½ the sample (50 µl) for the lowest three TPN levels to $\frac{1}{10}$ the sample for the highest level. Concentrations are given as 10^{-9} M (10^{-13} moles).

TPN^+	Yield* (60 min)	TPN^+	Yield* (30 min)	DPN^+	Yield† (30 min)
0.90	8000	29.2	5800	2.02	3710
0.90	7810	29.2	5780	2.02	3760
0.90	7810	29.2	5640	2.02	3450
0.90	8020	29.2	5780	2.02	3580
2.86	8000	93.5	5320	3.98	3710
2.86	8110	93.5	5210	3.98	3450
2.86	8000	93.5	5250	3.98	3450
2.86	8000	93.5	5300	7.90	3250
9.16	7610	298	2300‡	7.90	3320
9.16	7700	298	2340‡	7.90	3280
9.16	7740	298	2320‡	7.90	3360
9.16	7900	298	2320‡		

* Ratio of 6-P-gluconate produced to TPN present.

† Ratio of pyruvate produced to DPN present.

‡ On 15-minute incubation.

ASSESSMENT OF PROCEDURES

Reproducibility and Proportionality—The cycling procedures are reproducible almost to within the limits imposed by the fluorometer (Table I, Fig. 2). The data presented in Table V represent 36 analyses for each of the nucleotides. The pooled standard deviations for DPN^+, DPNH, TPN^+, and TPNH were 2.0, 2.2, 2.9, and 1.9%, respectively.

The proportionality between TPN concentration and 6-P-gluconate formation is quite satisfactory and permits assay of a 100-fold range of coenzyme with the same cycling reagent (Table II). The yield of pyruvate from DPN^+ is less proportional. Without serious departure from linearity the practical limit for a given cycling reagent and incubation time is a 20-fold range, *i.e.* conversion of 0.005 to 0.1% of lactate to pyruvate. With sufficient numbers of standards the range can nevertheless be extended. DPN was added in steps of two from 1.8×10^{-8} to 5.8×10^{-7} M. Successive higher levels compared to the lowest level were, respectively, 93, 83, 70, 56, and 44% of that expected for proportionality.

Specificity—When added to the TPN cycle, DPN at 10 times the TPN level neither gives a significant blank nor alters the cycling rate for TPN (Table III). Similarly, TPN does not interfere in the DPN cycle with TPN:DPN ratios of 100:1 (Table III). When TPN^+ at a concentration of 5×10^{-6} M was heated in 0.02 N H_2SO_4 for 30 minutes at 60°, not more than 1 part in 2000 was converted to DPN^+ (measured by cyling). Some decrease was found in the cycling rate for TPN in the presence of high concentrations of the alkali degradation products of TPN^+. TPN at 5×10^{-8} M was inhibited 16 and 33%, respectively, by 2×10^{-6} M and 10^{-5} M TPN^+ that had been destroyed with alkali. On the other hand, the cycling of 2×10^{-7} M DPN was inhibited less than 10% by 4×10^{-5} M DPN^+ that had been destroyed with alkali.

Whole homogenates of liver, brain, and blood, treated successively with acid and alkali to destroy all pyridine nucleotides, contribute blank values equivalent to 10^{-6} to 10^{-7} moles of DPN

or TPN per kg of tissue (Table IV). These blanks are negligible in relation to the native levels of the pyridine nucleotides (Table VI) with the possible exception of TPN^+ in liver and brain. In this case, the tissue blanks approximate 2 and 5%, respectively, of the relatively low amounts of TPN^+ present.

Low levels of DPN and TPN were added back to acid-alkali-treated tissue samples. Recovery was complete (Table IV). Thus, no tissue constituents stable to both acid and alkali interfere when present at tissue levels as high or higher than those required for measuring native nucleotides (Table VI).

TABLE III

Tests for possible interference of one nucleotide with measurement of the other

DPN^+, TPN^+, and combinations of the two nucleotides were added to complete cycling mixtures designed for DPN or TPN assay. The "observed" values are based on comparison of observed pyruvate or 6-P-gluconate produced, with the amounts formed by each nucleotide when used alone in its proper cycling mixture. All values are recorded as 10^{-8} moles per liter of cycling mixture.

DPN cycle			TPN cycle			TPN cycle		
Present		Observed	Present		Observed	Present		Observed
TPN^+	DPN^+	DPN^+	DPN^+	TPN^+	TPN^+	DPN	TPN	TPN^+
5	4.65	4.80	9	0.91	0.85	9	0	−0.01
50	4.65	4.73	90	0.91	0.94	90	0	0.00
5	0	<0.1	90	9.33	9.35	900	0	0.07
50	0	0.2	900	9.33	9.05			

TABLE IV

Tests for possible interference in DPN and TPN measurements in tissues

The tissues were homogenized in 0.02 N HCl, then made 0.02 N in NaOH (excess), and heated for 10 minutes at 100° to destroy all pyridine nucleotides. Known amounts of DPN^+ or TPN^+ were then added back as indicated. Cycling was carried out with whole homogenates. All analyses were made at 100 μl cycling volume in triplicate. All values are recorded as micromoles per kilogram of fresh brain or liver or per liter of blood.

Tissue	DPN		TPN	
	Calculated	Found	Calculated	Found
Liver (μg)				
22	0	<3		
22 + DPN^+	207	207		
16			0	2.3
16 + TPN^+			25.8	28.1
Brain (μg)				
60	0	<1		
60 + DPN^+	75	74		
100			0	0.4
100 + TPN^+			4.7	5.1
Blood (μl)				
0.08	0	<1		
0.08 + DPN^+	57	58		
1.2			0	0
1.2 + TPN^+			3.6	3.8

TABLE V

Recovery of pyridine nucleotides added to homogenates of liver and brain and to diluted blood

Tissue samples (200 mg) were homogenized in 20 ml of ice-cold 0.02 N H_2SO_4-0.1 M Na_2SO_4 or 0.02 N NaOH-5 × 10^{-4} M cysteine. Blood was diluted 1:100 in the same acid solution or in 0.1 M Na_2CO_3-0.02 M $NaHCO_3$. The samples were heated at 60° for either 45 minutes (acid) or 10 minutes (alkaline). The pyridine nucleotides were added before heating. Standards consisted of DPN^+ and TPN^+, and were prepared and heated in H_2SO_4-Na_2SO_4 like the samples. Assays were conducted in triplicate at 100 μl volume with amounts of tissue indicated. All values are recorded as 10^{-6} moles per kg wet weight or per liter.

Tissue	DPN^+		DPNH		TPN^+		TPNH	
	Calculated	Found	Calculated	Found	Calculated	Found	Calculated	Found
Liver (μg)								
1		640						
1 + DPN^+	2690	2620						
4				215		172		455
4 + DPNH			723	644				
4 + TPN^+					657	630		
4 + TPNH							935	895
Brain (μg)								
2		308						
2 + DPN^+	1310	1360						
10				97				
10 + DPNH			294	293				
20						7.0		25.4
20 + TPN^+					58.1	56.0		
20 + TPNH							118	113
Blood (μl)								
0.006		132						
0.006 + DPN^+	451	435						
0.02				96		14.8		15.6
0.02 + DPNH			198	201				
0.02 + TPN^+					66.2	64.9		
0.02 + TPNH							114	115

Analysis of Tissues for Pyridine Nucleotides—The difficulty of preparing fresh tissues for pyridine nucleotide analysis has been mentioned. The preparation procedure suggested above was tested by recovery experiments from liver, brain, and blood. The nucleotides were added before heating the acid or alkaline homogenates or dilutions to 60°. Recovery was satisfactory in all cases (Table V). To test more rigorously whether nucleotide-destroying enzymes had been removed, additional samples were brought to pH 8 and incubated 30 minutes at 38° before cycling at 50-fold greater tissue concentrations than those used during cycling. The samples that had been pretreated with alkali (for DPNH and TPNH assay) were unaffected by this incubation near neutrality.[3] However, samples pretreated with acid (for assay of DPN^+ and TPN^+) still contained DPNase. The losses from liver, brain, and blood for DPN^+ were 35%,

[3] Since these samples contained only TPNH and DPNH, the possibility that some DPN- or TPN-destroying activity might remain was not ruled out, but separate experiments demonstrated the absence of DPN^+-splitting activity.

TABLE VI

Analysis of rat liver brain and blood for pyridine nucleotides

Samples were obtained from four male rats, 5 months old (Sprague-Dawley) and prepared for analysis as described in Table IV. All the blood possible was removed through the right heart. Liver samples were homogenized approximately 3 minutes postmortem, brain samples approximately 5 minutes postmortem. The whole treated homogenates, suitably diluted, were analyzed at a cycling volume of 100 μl in triplicate. The respective amounts of liver and brain per sample were 3 and 6 μg for DPN$^+$, 10 and 30 μg for both DPNH and TPN$^+$, and 4 and 30 μg for TPNH. A volume of 0.015 μl of blood was used for DPN$^+$ and 0.03 μl for the rest of the assays. All values are recorded as 10^{-6} moles per kg wet weight or per liter.

	DPN$^+$	DPNH	TPN$^+$	TPNH	Total DPN	Total TPN
Liver, rat No. 1	674	279	128	567	953	695
Liver, rat No. 2	575	230	111	491	805	602
Liver, rat No. 3	648	230	112	401	878	513
Liver, rat No. 4	617	270	108	549	887	657
Average	628	252	115	502	880	617
Brain, rat No. 1	321	94	6.9	21.3	415	28
Brain, rat No. 2	354	92	7.5	22.6	446	30
Brain, rat No. 3	287	87	7.3	22.4	374	30
Brain, rat No. 4	324	108	7.1	22.8	432	30
Average	322	95	7.2	22.3	417	30
Blood, rat No. 1	120	90	12.0	8.9	210	21
Blood, rat No. 2	121	80	14.2	9.0	201	23
Blood, rat No. 3	98	85	13.5	15.3	183	29
Blood, rat No. 4	99	94	15.7	12.7	193	28
Average	110	87	13.8	11.5	197	25

35%, and 0, respectively, and for TPN$^+$, 72%, 90%, and 20%, respectively. The fact that recoveries were satisfactory in the analyses of Table V is attributed to the dilution during cycling which prevented important destruction by residual enzyme activity.

As a demonstration of the use of the cycling procedures, liver, brain, and blood from four rats were assayed for the four nucleotide forms (Table VI). The values for DPN$^+$ and DPNH for liver are close to those reported by Glock and McLean (14) and by Jacobson and Kaplan (15) and somewhat higher than those found by Helmreich *et al.* (16) and Bassham *et al.* (17). The TPN$^+$ and TPNH values, however, are approximately twice those reported by the above workers (14, 15, 17). The total DPN values in brain and blood are approximately 50% greater than those found by Glock and McLean (14) and the total TPN values are nearly double their figures. The TPN$^+$ levels of Table VI in particular are higher than those in the literature.[4] The higher values appear to be the result of changes in preparation of tissues for analysis (greater initial dilution, circumvention of DPNase destruction, prevention of oxidation and, therefore, loss of reduced nucleotides by hemoglobin, and use of whole tissue rather than extracts).

[4] Preliminary results with frozen-dried material indicate that TPN$^+$ levels in the living tissue are in fact much higher than those recorded in Table V. Apparently, there is an exceedingly rapid reduction of TPN during the ordinary process of homogenization.

DISCUSSION

The cycling systems described were chosen from among many possibilities. The positive factors in the choice of each system included (*a*) commercial availability of the required enzymes, (*b*) favorable kinetic possibilities, (*c*) stability and nonvolatility of the substrates or products, and (*d*) ready measurement of one of the products by a pyridine nucleotide system.

The TPN system is the better of the two. Glutamic dehydrogenase represents one of the few easily available enzymes for oxidizing TPNH and it is used in the kinetically and thermodynamically favorable direction. Old yellow enzyme is another possibility and might have advantages since oxygen would be the only substrate required. Glucose-6-P dehydrogenase is ideal as the other enzyme component since the reduction of TPN$^+$ is irreversible after hydrolysis of 6-P-gluconolactone and since the product is easily measured by 6-P-gluconate dehydrogenase.

The DPN system is not ideal because it lacks an irreversible step, and because of the unfavorable lactate-pyruvate equilibrium. Several other systems were considered. Alcohol dehydrogenase was used earlier coupled with malic dehydrogenase. High cycling rates were obtained, but the system was discarded because of the volatility of both alcohol and acetaldehyde. Possibly, the same system used with a longer chained alcohol would have merit. Glycerol-P dehydrogenase was explored briefly as the oxidizing enzyme coupled with lactic dehydrogenase plus pyruvate. This system was abandoned because of the instability of dihydroxyacetone-P, although the kinetics is quite favorable. Successful cycling was obtained with the glutamic dehydrogenase-lactic dehydrogenase system operating in the opposite direction from that proposed here. The over-all equilibrium constant is more favorable, but the cycling rate is low due to the low velocity of glutamate oxidation. Nevertheless, the system may have real advantages if a cycling rate of 500 per hour is sufficient. The α-ketoglutarate formed is readily measured with TPNH which would permit double cycling through the TPN system.

In the proposed DPN system it is possible that skeletal muscle lactic dehydrogenase could be substituted for heart lactic dehydrogenase. The Michaelis constant for DPN$^+$ is higher, and, therefore, the first order reaction constant lower, than with the heart muscle enzyme, but more enzyme could be used to compensate. Commercial preparations gave high blank values, presumably due to contamination with DPN$^+$. If this could be removed with charcoal, skeletal muscle lactic dehydrogenase would probably be quite satisfactory.

Either of the cycling systems described can be repeated to give over-all multiplication of 10^7 to 10^8. To exploit fully such multiplications it would be necessary to carry out the first cycling in a very small volume. Without cycling it is possible to measure 10^{-11} moles of pyridine nucleotide in 1 ml final volume, *i.e.* at a concentration of 10^{-8} M. A double cycling with 10^8 multiplication would extend this sensitivity to 10^{-19} moles. This is the equivalent of 0.000,1 μl of a 10^{-9} M solution (the present lower limit of concentration for satisfactory reproducibility). One molecule of an enzyme with turnover number of 10,000 per minute would produce 10^{-18} moles of product in an hour. If the reaction could be made to result in oxidation or

reduction of an equivalent amount of pyridine nucleotide, this would be measurable by double cycling if the first cycling step could be carried out in 0.001 μl or less.

The advantage of the cycling methods is not limited to an increase in sensitivity. The multiplication process is capable of eliminating many disturbing factors. For example, in spite of a very unfavorable equilibrium constant, lactate can be nearly quantitatively converted to pyruvate with lactic dehydrogenase by a 200-fold excess of DPN^+ at pH 10.7. If the DPNH were to be measured directly, blank fluorescence from DPN^+ at this pH value would be very disturbing. However, after destroying excess DPN^+ with stronger alkali, the DPNH can be measured by cycling with no DPN^+ blank.

The cycling procedures make it practicable to measure tissue concentrations as low as 10^{-6} M of any substrate that can be directed to a DPN or TPN system and which will stand mild exposure to acid or alkali. Such measurement is possible without concentrating the substrate or preparing an extract. The tissue homogenate is first pretreated with acid or alkali to destroy native nucleotides and enzymes, after which the appropriate enzyme system and pyridine nucleotide are added. The excess nucleotide is then destroyed with acid or alkali, as the case may require, and the nucleotide that has been formed by oxidation or reduction is measured by a cycling method.

SUMMARY

1. Methods are presented for the measurement of as little as 10^{-15} moles of diphosphopyridine or triphosphopyridine nucleotide. This is an increase in sensitivity over present methods of several thousand fold.

2. Triphosphopyridine nucleotide (oxidized or reduced) at a concentration of 10^{-9} to 3×10^{-7} M is made to catalyze the enzymatic dismutation of α-ketoglutarate, NH_4^+, and glucose 6-phosphate to glutamate and 6-phosphogluconate. The 6-phosphogluconate produced is measured in a second step with 6-phosphogluconate dehydrogenase and triphosphopyridine nucleotide added in excess.

3. Diphosphopyridine nucleotide at levels as low as 10^{-9} M is made to catalyze the dismutation of α-ketoglutarate, NH_4^+, and lactate to glutamate and pyruvate. The pyruvate produced is measured in a second step with lactic dehydrogenase and an added excess of reduced pyridine nucleotide.

4. If greater sensitivity is required the cycling processes can be repeated with an over-all yield of 10^7 to 10^8. This would suffice to measure 1 molecule of any enzyme which forms a product that can be led to a pyridine nucleotide system.

5. The use of the cycling procedures is illustrated by analyses of rat liver, brain, and blood for oxidized and reduced nucleotides. Because of the sensitivity it was unnecessary to prepare extracts of the tissues. Higher values than those reported previously were observed for oxidized and reduced triphosphopyridine nucleotide. These higher values are attributed to changes in tissue preparation, some of which were made possible by the sensitivity of the cycling methods.

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