A Dimer of Diphosphopyridine Nucleotide*

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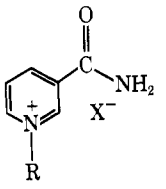
ABSTRACT: The existence of a dimeric form of diphosphopyridine nucleotide was postulated in order to interpret the results of previous electrochemical studies. A salt of this dimer has now been isolated. Direct molecular weight determinations by vapor pressure osmometry and by sedimentation equilibrium measurements in the ultracentrifuge have confirmed the dimeric nature of the material. Chromatography on a Sephadex column and polarographic estimates of diffusion coefficients furnish additional support. The structure of the dimer has not been conclusively proved; the ultraviolet and nuclear magnetic resonance spectra suggest that dimerization may occur at the 4 position of the pyridine ring. The electrochemical preparation of the dimer at a cathode of controlled potential is clean and quantitative. An enzyme in etiolated mung bean seedlings is capable of oxidizing the dimer to oxidized diphosphopyridine nucleotide. Confirmation of the dimeric species permits a stronger position regarding certain points of incoherence and confusion in the literature.

1-Substituted nicotinamide salts (I), including the



I

biologically important pyridine coenzymes DPN^+[1] and TPN^+, exhibit two polarographic reduction waves. The first represents a reversible one-electron process which is followed by an irreversible chemical step, while the second corresponds to a two-electron reduction to the 1,4-dihydropyridine (Burnett and Underwood, 1965a,b; Cunningham and Underwood, 1966, 1967). In the case of 1-methylnicotinamide, the formation of a dimer as the final product at a potential on the first wave was confirmed by a molecular weight determination (Burnett and Underwood, 1965a), while with DPN and TPN it was inferred from indirect evidence (Burnett and Underwood, 1965b; Cunningham and Underwood, 1966, 1967). Cyclic voltammetry with fairly fast voltage scans disclosed the generation of free radicals on the first wave with a half-life of the order of perhaps 1 msec (Cunningham and Underwood, 1967). Dimerization of the radicals, while consistent with all of the data, was essentially not proved in the case of the coenzymes.

Several types of direct evidence have now been obtained which vindicate the postulation of a dimer. Sedimentation equilibrium measurements in the ultracentrifuge, performed on solutions after complete electrolysis at a potential on the first wave, have yielded a value consistent, in view of expected errors, with formulation of the DPN species in solution at pH 10 as a dimeric tetraanion. The dimer has been crystallized as the Tris salt from aqueous acetone. Measurements on solutions of this salt in the vapor pressure osmometer have given a satisfactory molecular weight when adjusted for the number of ions furnished by Tris$_4$dimerate$\cdot 6H_2O$. Estimated diffusion coefficients obtained from the cathodic wave of DPN^+ and the anodic wave of the electrolytic reduction product suggest that the product is a much larger molecule than DPN^+. The product is eluted from a column of Sephadex G-15 considerably faster than is monomeric coenzyme, again suggesting that a larger molecule is involved. The ultraviolet and nuclear magnetic resonance spectra suggest that dimerization may occur at the 4 position of the pyridine ring, although this conclusion is considered tentative at this time. The discussion below will show that discordant statements in the literature can be reconciled and rationalized by the acceptance of the dimer.

Experimental Section

Materials. β-DPN^+ and DPNH were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. The former was purified by chromatography on DEAE-cellulose and crystallization of the free acid from aqueous acetone (Winer, 1964). Coenzyme solutions were assayed by published methods (Colowick and Kaplan, 1955). Mung bean seeds were purchased from Johnson Seed Co., Enid, Okla. Yeast alcohol dehydrogenase was obtained from Worthington Biochemicals, Freehold, N. J. Reagent

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[1] Abbreviations used are listed in *Biochemistry 5*, 1445 (1966).

grade chemicals and deionized water were used throughout.

Apparatus. The polarograph was conventional. The apparatus for controlled potential coulometry comprised a Wenking Model 61TRS potentiostat, a coulometer described in the literature (Wise, 1962; Bard and Solon, 1962), and the same type of three-electrode cell used before (Burnett and Underwood, 1965a). Ultraviolet spectra were recorded with a Cary Model 14 spectrophotometer, fluorescence spectra with a Farrand spectrofluorometer with automatic scanning, and nuclear magnetic resonance spectra with a Varian Model A-60-A instrument. Sedimentation equilibrium experiments were performed with a Spinco Model E ultracentrifuge with an An-E-rotor. Refractive index measurements required for the ultracentrifuge study were made with a Brice–Phoenix differential refractometer at 546 mμ. pH values were determined with a Radiometer Model 26 pH meter. Molecular weight measurements were performed using a Mechrolab Model 301A vapor pressure osmometer with an aqueous thermistor probe and a 37° thermostat.

Dimer Preparation. The half-wave potential for the first reduction wave of DPN^+ is −0.93 V *vs.* saturated calomel electrode, while the second wave occurs at about −1.7 V (Burnett and Underwood, 1965b). There is a broad plateau where reduction to the dimer occurs with 100% current efficiency. DPN^+ solutions, usually 10^{-3}–10^{-2} M, were electrolyzed at a large mercury pool cathode controlled at −1.2 to −1.3 V *vs.* saturated calomel electrode. The reduction was monitored coulometrically; as before (Burnett and Underwood, 1965b), exhaustive electrolysis always yielded a quantitative one-electron reduction. An atmosphere of prepurified nitrogen was maintained. The dimer samples for the ultracentrifuge experiments were prepared in a 0.1 M carbonate buffer of pH 10; a Tris buffer of pH 9 was used where the dimer was to be crystallized; in certain other cases, phosphate and pyrophosphate buffers were employed.

Ultracentrifuge Experiments. A 30-mm double-sector cell was employed with 0.25 ml of freshly prepared dimer solution (or a DPN^+ solution, for comparison) in one side and 0.30 ml of buffer in the other. Sedimentation was followed with a Rayleigh interference optical system at 546 mμ. The rotor was run at 26,000 rpm for about 1 hr and then at 24,000 rpm until equilibrium was attained, usually about 12 hr. The technique was essentially conventional. Data analysis followed the treatment of Johnson *et al.* (1959) for charged solutes. Computations were performed using a modification of a FORTRAN program previously described (Esval, 1962; Esval and Johnson, 1965).

Crystallization of the Dimer. About 1 g of commercial DPN^+ was eluted from a column of Whatman DE-11 DEAE-cellulose with a Tris buffer concentration gradient at pH 7.0 (Winer, 1964). About 200 ml of solution, representing the central 80% of the DPN^+ band, was adjusted to pH 9.0 with tetra-*n*-butylammonium hydroxide and electrolyzed as above. The usual potassium chloride anolyte in the silver–silver chloride auxiliary electrode compartment was replaced with Tris-Cl so that leakage of electrolyte would not introduce a salt less soluble than Tris-Cl in acetone. The electrolyzed solution was transferred to a cold room at −15°, and 1.5 l. of precooled acetone was added dropwise with continuous mechanical stirring. This was essentially the same crystallization procedure as that of Winer (1964) for DPN^+ except that the solution was not acidified before the addition of acetone. The precipitated Tris salt of the dimer was allowed to age for several hours at −15°, the supernatant solution was decanted, and the yellow solid was collected on a "fine" sintered-glass filter, washed several times with acetone at −15°, and dried overnight *in vacuo* over phosphorus pentoxide, yield 850 mg. *Anal.*[2] Calcd for Tris$_4$dimerate·$6H_2O$: C, 36.25; H, 5.87; N, 13.12. Found: C, 38.01; H, 5.99; N, 12.83.

Vapor Pressure Osmometry. Measurements were performed in the usual manner with solutions of the Tris dimer salt in water. The instrument was calibrated with standard solutions of sucrose.

Polarography. A polarogram was obtained on a 7×10^{-4} M solution of DPN^+ in 0.1 M phosphate buffer of pH 7.4. The solution was then electrolyzed at −1.3 V *vs.* saturated calomel electrode at a large mercury pool cathode using the potentiostat, and a second polarogram was recorded. The temperature and the capillary characteristics of the dropping mercury electrode were the same in both cases; every effort was made to control factors affecting the diffusion current so as to obtain as closely as possible a comparison of the diffusion coefficients of DPN^+ and the dimer.

Gel Filtration. A column of Sephadex G-15 (12 × 350 mm) was prepared in 0.1 M phosphate buffer at pH 7.4, and 0.5 ml of the solution under study was applied to the top of the column. A flow rate of 0.6 ml/min of the buffer was maintained, the effluent was monitored at 280 mμ, and a signal proportional to transmittance was recorded *vs.* time.

Mung Bean Extraction. About 15 g of etiolated seedlings from 2 to 5 days old were homogenized in 100 ml of 0.1 M phosphate buffer of pH 7.4 in a Waring Blendor. The suspension was centrifuged at 27,000*g* for 15 min, and 50 ml of the clear supernatant solution was applied to a 20 × 500 mm column of Sephadex G-25. Upon elution with the same buffer, a turbid fraction appearing immediately after the void volume contained the desired enzyme activity, separated from a certain cofactor (see below). A solution containing the cofactor but no enzyme activity was obtained by boiling the supernatant solution from the centrifugation.

Stability Studies. Dimer solutions, maintained under various conditions of pH, temperature, and oxygenation, were measured at 340 mμ in timed experiments.

Nuclear Magnetic Resonance Spectrum. A solution of the dimer was prepared as described above. The solution was lyophilized, and the residue was taken up in D_2O for nuclear magnetic resonance examination.

[2] Analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind., and Spang Microanalytical Laboratory, Ann Arbor, Mich.

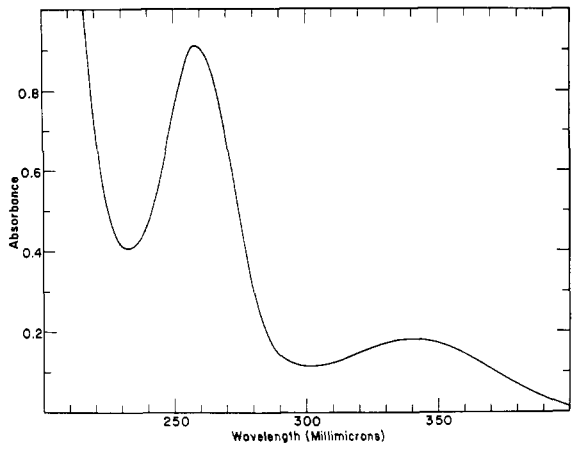


FIGURE 1: Ultraviolet absorption spectrum of DPN dimer. A 2.75×10^{-5} M solution of freshly prepared dimer in 0.1 M Tris buffer of pH 9.0 was used.

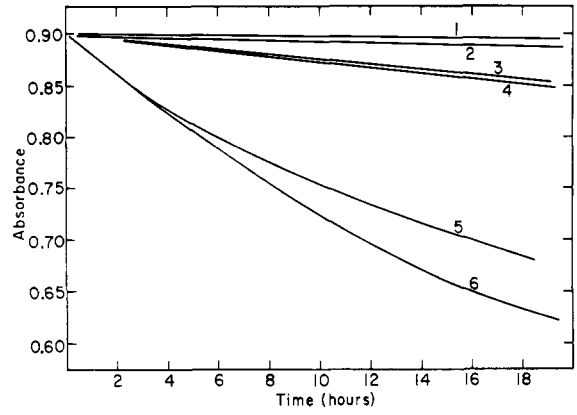


FIGURE 2: Stability of dimer solutions. Absorption was at 340 mμ *vs.* time; buffer, pH, temperature, and saturating gas, respectively, are given in curve listings. Curve 1: borate, 10.0, 3°, air; curve 2: borate, 10.0, 25°, air; curve 3: phosphate, 7.4, 3°, N_2; curve 4: phosphate, 7.4, 3°, air; curve 5: phosphate, 7.4, 25°, N_2; and curve 6: phosphate, 7.4, 25°, air.

Results

Ultracentrifuge. The molecular weight of the anhydrous dimeric tetraanion of DPN is theoretically 1324. After preliminary runs in which various difficulties were overcome, an experiment under optimal conditions yielded a value of 1120. The experiment was performed in a buffer of pH 10.0, representing a compromise regarding the well-known instability of DPN^+ at high pH values and that of the dimer at lower ones. In view of the duration of the preparative electrolysis and the sedimentation equilibrium experiment, the limited stability of the compounds, the low molecular weights, and the uncertain hydration of the species, it is doubtful that closer agreement with the theoretical molecular weight can be expected. (The instability problem might appear circumventable by working at a lower temperature, but in one experiment with the rotor refrigerated to 8°, a value of 1870 was obtained, perhaps suggesting weak association at this temperature.) Monomeric DPN^+ yielded a value of 789 (theoretical anhydrous molecular weight 663). The charge on the molecule enters into the calculation of the molecular weight by this method. The pK_a of DPN^+ is about 3.8 (C. E. Moore, Jr., and A. L. Underwood, unpublished data). Thus it appears safe to assume a charge of -1 for DPN^+ and -4 for the dimer at pH 10, and the calculations were performed on this basis. The structural change upon reduction and dimerization would surely not affect the phosphate groups or the adenine moiety sufficiently to endanger this assumption.

Vapor Pressure Osmometry. Duplicate experiments with aqueous solutions of the Tris salt gave 2054 and 2038 (average, 2046) for the molecular weight on the assumption of five particles per molecule. The calculated value for Tris$_4$dimerate$\cdot 6H_2O$ is 1922. Errors of 3–5% in osmometry are usual, in our experience.

Polarography. The limiting current for the reduction process of DPN^+ to dimer is diffusion controlled (Burnett and Underwood, 1965b). Similarly, in the case of the anodic wave for solutions of the dimer ($E_{1/2}$ = -0.25 V *vs.* saturated calomel electrode), it is found that the wave height varies with the square root of the corrected mercury height, suggesting diffusion control for this process as well. Thus the Ilkovic equation is pertinent for both processes, and the ratio of the squares of the diffusion currents for the two species reduces (with other factors controlled) to a ratio of diffusion coefficients

$$\frac{i_d^2{}_{DPN^+}}{i_d^2{}_{dimer}} = \frac{(607nCm^{2/3}t^{1/6})^2\, D_{DPN^+}}{(607nCm^{2/3}t^{1/6})^2\, D_{dimer}} = \frac{D_{DPN^+}}{D_{dimer}}$$

where D is the diffusion coefficient and the other symbols have their usual polarographic significance.

The value found experimentally for this ratio in the present case is 1.95. Hydrodynamic theory for spherical molecules predicts a diffusion coefficient inversely proportional to the radius of the molecule (Bird *et al.*, 1960). Thus the data suggest that the dimer has an effective radius roughly twice that of DPN^+. Although the approximation of sphericity is admittedly crude, it should be noted that there is evidence that DPN^+ is folded upon itself in solution, with a significant interaction of the nicotinamide and adenine portions of the molecule (Kaplan, 1960). One should be cautious with these data, but in concert with the other evidence they support the existence of the dimer.

Gel Filtration. The elution volume for DPNH was about 1.4 times that for the dimer on a column of Sephadex G-15. Normal, symmetrical peaks with no tailing were obtained in both cases. An accurate molecular weight determination by this means requires calibration with a series of closely related compounds (preferably oligomers), which are not at hand. However, the experiment suggests that the dimer is significantly larger than DPN itself. The reduced coenzyme was employed for the comparison because structurally it is somewhat more closely related to the dimer than is the zwitterionic DPN^+.

Ultraviolet Spectrum. Figure 1 shows the spectrum of

a fresh preparation of the dimer diluted to 2.75×10^{-5} M in a Tris buffer of pH 9.0. The two bands occur at essentially the same wavelengths as those of DPNH. The values are λ_{max} 259 mμ (ε 33,100) and 340 mμ (ε 6550). These molar absorptivities may be compared with literature values for monomeric DPNH of 14,400 and 6220 (Horecker and Kornberg, 1948; Siegel *et al.*, 1959).

Nuclear Magnetic Resonance Spectrum. The nuclear magnetic resonance spectrum of the dimer was closely similar to the published spectrum of DPNH (Meyer *et al.*, 1962). The only appreciable difference was the broadening and splitting, with the dimer, of the band assigned by Meyer *et al.* to the proton at the 4 position of the pyridine ring.

Fluorescence. Solutions of the dimer in Tris buffer of pH 9.0 exhibited no fluorescence between 220 and 700 mμ with excitation of 220–700 mμ. This contrasts with the behavior of DPNH, which is strongly fluorescent, suggesting a mode of intramolecular quenching unavailable in the reduced coenzyme.

Stability of Dimer Solutions. Thorough study of the dimer stability has not been completed, but some preliminary measurements were performed to aid in the isolation and handling for other experiments. The results are shown in Figure 2.

Enzymatic Oxidation of Dimer. The dimer was oxidized to DPN^+ by the mung bean enzyme in the presence of a heat-stable factor in the bean seedlings. Details need not be given because the results are essentially the same as those obtained by Kono and Suekane (1958). The reader should note, however, that Kono incorrectly considered the dimer to be a dihydropyridine isomeric with DPNH. Kono clearly showed, by enzymatic assays, that the oxidation product of the dimer was DPN^+; this has been confirmed in the present study. The enzyme catalyzing the dimer oxidation was partially purified by differential precipitation with ammonium sulfate and by electrophoresis; the oxidant (Kono's cofactor) was not identified (Kono and Suekane, 1958).

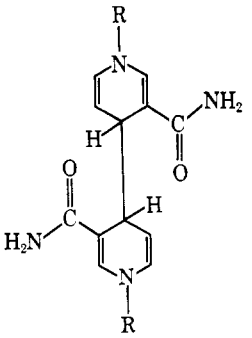
Discussion

The evidence for the formation of a dimer in electrolysis at a potential on the first reduction wave of DPN^+ may be summarized. (1) Polarography, controlled potential coulometry, and cyclic voltammetry indicate that the product is formed by a reversible one-electron transfer which is followed by an irreversible chemical step (Burnett and Underwood, 1965b; Cunningham and Underwood, 1967). (2) The product possesses neither DPN^+ nor DPNH coenzyme activity in the conventional assay with yeast alcohol dehydrogenase (Burnett and Underwood, 1965b). (3) The model compound 1-methylnicotinamide definitely yields a dimer during electrolysis on the first wave (Burnett and Underwood, 1965a). (4) Sedimentation equilibrium measurements in the ultracentrifuge yield a molecular weight much too high for a monomeric DPN species but reasonable for a dimer in the light of experimental difficulties. (5) The diffusion coefficient from polarographic measurements is much smaller than that of monomeric DPN^+. (6) The product is eluted much more rapidly than is monomeric DPNH in gel filtration chromatography on a Sephadex preparation which fractionates up to mol wt 1500.

The results of the carbon determination in the elemental analysis of the Tris salt of the dimer do not agree well with the theoretical value. Adjusting the number of molecules of water of hydration obviously does not improve the situation; as agreement is approached for per cent carbon, the calculated and observed hydrogen and nitrogen values diverge. To test the possibility of an analytical idiosyncrasy in this type of compound, a sample of $DPN^+ \cdot 3H_2O$, carefully prepared by the purification and crystallization procedure of Winer (1964), was analyzed. (This material has been cited as possibly the ultimate in DPN^+ purity; Chaykin, 1967.) *Anal.* Calcd: C, 35.16; H, 4.65; N, 13.67. Found: C, 38.42; H, 4.46; N, 13.82. Thus it is possible that a difficulty does exist in the carbon determination. On the other hand, a satisfactory elemental analysis of DPN^+ was reported by Winer (1964), and it may be that the real problems are the uncertain hydration of the dimer, the lability generally associated with partially reduced pyridine derivatives, or contamination of the precipitated dimer salt. In any event, failure of the elemental analysis totally to support the purity of the isolated dimer salt hydrate surely cannot jeopardize the preponderant physicochemical evidence for the existence of the dimer in solution.

The dimer of DPN reported here is apparently the same reduction product obtained when oxygen-free DPN^+ solutions containing ethanol are irradiated with X-rays. Swallow first reported the product to be DPNH (Swallow, 1953), but later work showed that, although an absorption band at 340 mμ was present, the product differed from DPNH in being nonfluorescent and in lack of coenzyme activity with lactic and alcohol dehydrogenases (Swallow, 1955). Stein and Swallow (1958) found, by careful study of the reduction yield and by stoichiometric analysis of the reaction of the product with methylene blue, that it was one electron removed from DPN^+ per nicotinamide unit. It was suggested that DPN^+ was reduced by hydroxyethyl radicals to a free-radical species which dimerized.

Unequivocal evidence for the structure of the dimer is unfortunately not at hand. A rigorous analysis of the nmr spectra of the pyridine coenzymes is not possible at this time; for example, the ribose protons give an uninterpretable series of overlapping multiplets which obscure much of the region of interest. However, protons at the 2 and 8 positions of the adenine moiety and the 2 and 4 positions of the nicotinamide ring give resonance lines which are well separated from the more confusing portion of the spectrum (Meyer *et al.*, 1962). Because the major difference between the nuclear magnetic resonance spectrum of the dimer and that of DPNH is the broadening and splitting, with the former, of the band assigned by Meyer *et al.* to the protons at the 4 positions of the pyridine rings, the nuclear magnetic resonance spectra suggest that dimerization occurs at the 4 positions (II). The protons at these positions in a 4,4′ dimer would be in similar, but not necessarily equivalent, environments, and could thus be spin coupled to each other and to neighboring ring protons, giving rise



II

to a series of overlapping multiplets which could explain the broadening of the band.

The ultraviolet spectrum of the dimer represents less than conclusive evidence regarding the position of dimerization. Reasoning from the spectra of the three isomeric dihydropyridines reportedly obtainable by reduction of 1-substituted nicotinamides, Wallenfels and Gellrich (1959) have assigned structures to dimers of nicotinamide model compounds: dimerization at the 2 position of the pyridine ring should lead to a long-wavelength absorption band, at about 400 mμ; a 6,6′ dimer should exhibit two bands, at about 350 and 270 mμ; a 4,4′ dimer should exhibit one band, at about 350 mμ. (The exact wavelengths depend, of course, upon substituent groups.) So far as its utility for characterizing the pyridine portion of the molecule is concerned, the 270-mμ region is obscured in the case of DPN by the strong band at about 260 mμ characteristic of the adenine moiety.

Chaykin and Meissner (1964) and Chaykin *et al.* (1966) have reported the preparation of the 1,2-, 1,4-, and 1,6-dihydropyridines by borohydride reduction of DPN^+. Their ultraviolet spectra were comfortably similar to those expected on the basis of Wallenfels' work with model compounds. The 1,6-DPNH exhibited a band at 345 mμ, and (of course) a second band at 260 mμ. One reviewer of this paper pointed out that there is extra absorption at 260 mμ for the dimer (ε 33,100) as compared with two adenine moieties (2 × 14,400 = 28,800) if it be supposed that the adenine absorption is independent of the state of the nicotinamide portion of the molecule. This might imply absorption at two wavelengths (340 and 260 mμ) by the nicotinamide moiety of the dimer, in turn suggesting dimerization at the 6 position. Intriguing as this is, caution is required by the general state of structure–ultraviolet spectra correlations involving partially reduced pyridines. It may be noted that the dimer absorbs much less strongly at 340 mμ than would be expected of two 1,4-DPNH units (ε 6650 *vs.* 2 × 6220). This is not conclusive evidence against a 4,4′ dimer structure, however; for example, the probability of a transition introducing charge into the pyridine ring could be affected by the proximity of a second ring. It was found (Burnett and Underwood, 1965b) that the dimer was slowly reduced to DPNH at an electrode of sufficiently negative potential. This may strengthen the supposition that the dimer possesses the 4,4 structure, although possible isomerization of the double bonds beclouds this evidence. The present authors regard the 4,4′ structure as most likely for the dimer, but real proof of structure is not at hand.

There has been much confusion about the nature of the electrolytic reduction products of DPN^+. Kaye and Stonehill (1952) reported a single polarographic reduction wave; this is the same wave ($E_{1/2}$ = −0.927 V *vs.* saturated calomel electrode) as the first of the two waves found in this laboratory (Burnett and Underwood, 1965b). By comparing the diffusion current constant with that of acridine, Kaye and Stonehill erroneously reported that n = 2 for this wave, postulated two overlapping one-electron steps, and even calculated the formation constant for an intermediate semiquinone from polarographic potential data.

Ke (1956a) studied the polarography of DPN^+ and found only the first wave. The n value for this process was measured by passing the electrolysis current through a second polarographic cell containing cadmium ion (De Vries and Kroon, 1953), and the erroneous value n = 2 was obtained. The difficulties in this type of coulometry have been pointed out (Weaver and Whitnack, 1958). On the basis of his polarographic study, Ke suggested controlled potential electrolysis as a preparative method for DPNH (Ke, 1956a), but the attempt led to coenzymatically inactive material (Ke, 1956b). The product exhibited the same ultraviolet spectrum as the dimer reported here, and similarly failed to fluoresce. Misled by the incorrect n value, Ke concluded that the reduction product was very similar but not identical with enzymatically prepared DPNH (Ke, 1956b). In another paper (Ke, 1956c), partial conversion of DPN^+ to DPNH with platinum and lead electrodes at −1.4 V *vs.* saturated calomel electrode was reported. The DPN^+ reduction was not isolated from concurrent hydrogen evolution at these electrodes, and the results are difficult to interpret. In any case, it was suggested that the remaining, inactive reduction product might be an *o*-dihydropyridine isomeric with DPNH (Ke, 1956c); again, its properties clearly identify it as the dimer.

Powning and Kratzing (1957) reduced DPN^+ at a mercury electrode at −1.7 V *vs.* saturated calomel electrode and obtained about a 50% yield of DPNH. Again, it was suggested that an isomer of DPNH was produced, although dimerization was also mentioned as a possibility. At this potential, in our interpretation, both DPNH and dimer are formed (Burnett and Underwood, 1965b).

Likewise, Kono reported partial reduction to DPNH (Kono, 1957; Kono and Nakamura, 1958); again, the coenzymatically inactive portion of the reduction mixture resembled the dimer reported here. In a following paper (Kono and Suekane, 1958), it was stated that the reduction led to a mixture of the 1,2-, 1,4-, and 1,6-dihydropyridines. Interestingly, an enzyme system was found in etiolated mung bean seedlings which reoxidized the dimer to DPN^+, although of course it was incorrectly credited with the oxidation of the isomeric dihydropyridines (Kono and Suekane, 1958). Kono's observations have been confirmed in this laboratory. The dimer is oxidized to DPN^+ by a protein fraction

from mung beans in the presence of an additional, nonprotein factor which has not yet been characterized.

The repeated confusion of the DPN dimer with isomers of DPNH has led to an erroneous generalization regarding the modes of reduction of the pyridine coenzymes and nicotinamide model compounds. Pullman has attempted to show by molecular orbital calculations that reduction of DPN^+ by direct hydride ion transfer should yield the 1,4-dihydropyridine (DPNH) while reduction by a radical mechanism should form an *ortho* reduction product, either the 1,2- or the 1,6-dihydropyridine (*e.g.*, Pullman, 1964; Pullman and Pullman, 1963). The well-known work of Vennesland and others (*e.g.*, Vennesland, 1956) demonstrating a direct transfer of hydride ion from substrate to DPN^+ in redox reactions catalyzed by DPN-linked enzymes is correctly cited in support of the theoretical calculations, but the results of X-irradiation (Stein and Swallow, 1958) and electrolytic reduction (Powning and Kratzing, 1957) are incorrectly applied to vindicate the prediction that radical reduction leads to isomers of DPNH. It is clear that the dimer, not *o*-dihydro compounds, was obtained in these experiments. In the case of electrolytic reduction, DPNH *is* obtainable provided the electrolysis is performed, not on the first wave, but at a sufficiently negative potential. Pullman's use of the erroneous interpretation of the electrochemical reduction has been widely quoted (*e.g.*, Kaplan, 1960, p 118).

The dimer of DPN is easily prepared in quantitative yield by reduction at a potential on the first wave. Although its characterization has proved difficult, there is little doubt of its existence, and there is some evidence for its structure. The dimer should be taken into account by writers on the chemistry of pyridine compounds, and care should be taken not to confuse it with the two-electron reduction products. It is interesting that an enzymatic reaction of the dimer has been found. Of course it cannot be stated at present whether the dimer is a natural cofactor for this enzyme or whether the enzyme is simply nonspecific in this regard. There are no reports that indicate the occurrence of the dimer in living tissue, although it could easily be overlooked because of the similarity of its ultraviolet spectrum to that of DPNH. It will be interesting to test the activity of the dimer in a number of enzyme systems with various substrates.

Acknowledgments

At Emory University, Professor Morris Tager, Department of Microbiology, kindly made available an ultracentrifuge on which preliminary runs were made, Professor D. J. McCorquodale, Department of Biochemistry, provided cold room space, Professor Leon Mandell, Department of Chemistry, obtained nuclear magnetic resonance spectra, and Mr. C. E. Moore, Jr., prepared the purified DPN^+. We are deeply indebted to Dr. J. S. Johnson and Dr. R. M. Rush for the use of their ultracentrifuge facility at the Oak Ridge National Laboratory and for their generous help in the analysis of the data.

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