Electrochemical Reduction of Diphosphopyridine Nucleotide*

John N. Burnett† and A. L. Underwood

ABSTRACT: Diphosphopyridine nucleotide exhibits two polarographic reduction waves in tetra-*n*-butylammonium carbonate buffers, although several previous workers had observed only one. Analysis of the polarographic data and macroscale controlled potential coulometry indicate that at a cathode potential on the first wave diphosphopyridine nucleotide undergoes a reversible one-electron transfer process followed by an irreversible chemical step. Evidence suggests that the final product is a 4,4′ dimer of diphosphopyridine nucleotide arising from the combination of free radicals. This product exhibits an anodic wave and is reoxidizable to diphosphopyridine nucleotide at an electrode as well as by an enzyme obtained from mung beans, and it is electrolytically reducible to reduced diphosphopyridine nucleotide, but only at a very slow rate. At a cathode potential on the second wave, formation of reduced diphosphopyridine nucleotide and the above one-electron product occur simultaneously. Reduced diphosphopyridine nucleotide is electrolytically oxidizable to diphosphopyridine nucleotide. As a result of this study, similarities and differences in the electrochemical behavior of diphosphopyridine nucleotide and of certain nicotinamide model compounds are brought into sharper focus, and many anomalous reports in the literature can be rationalized.

There are several unresolved questions in the literature regarding the electrochemical reduction of diphosphopyridine nucleotide (nicotinamide-adenine dinucleotide, DPN).[1] For example, model compounds such as 1-methyl-3-carbamidopyridinium chloride (1-methylnicotinamide) exhibit two polarographic reduction waves (Tompkins and Schmidt, 1943; Sorm and Sormova, 1948; Ciusa *et al.*, 1950; Yasuda and Kitagawa, 1955; Nakaya, 1960; Burnett and Underwood, 1965), whereas DPN has been found to yield only one wave (Kaye and Stonehill, 1952; Carruthers and Suntzeff, 1953; Carruthers and Tech, 1955; Ke, 1956a) although one worker has reported two (Moret, 1955, 1956). Furthermore, attempts to prepare reduced diphosphopyridine nucleotide (DPNH) by electrolytic reduction of DPN^+ at a cathode of controlled potential have led to reduction products of variable coenzyme activity. The worst preparations were totally inactive (Ke, 1956b,c), while others exhibited 50% (Powning and Kratzing, 1957) and 65 to 76% of the expected DPNH activity (Kono, 1957; Kono and Nakamura, 1958). It is apparent that a reduction product other than DPNH may form in varying amount depending upon conditions, but neither its nature nor the manner of its formation has been elucidated in previous work.

In a recent paper (Burnett and Underwood, 1965) it was shown that the first reduction wave of 1-methyl-3-carbamidopyridinium chloride represented the uptake of one electron with the formation of free radicals which dimerized, probably at the 6 position of the pyridine ring. (The fact of dimerization was clearly established by a molecular weight determination; the structure of the dimer was inferred from its ultraviolet spectrum.) At a potential on the second wave, two electrons were taken up to form the 1,4-dihydropyridine, an analog of DPNH. The dimer did not function as an intermediate in the formation of the dihydro compound but rather was reducible in its own right to a product which was still dimeric.

In the present work, it has been found that two polarographic reduction waves may be reliably obtained with DPN under appropriate conditions. Reduction at a cathode potential on the first wave involves a reversible uptake of one electron followed by an irreversible chemical step. The product of this process is inactive by the usual enzymatic assays for DPN^+ and DPNH. It is believed that this product is a dimer and that, in contrast to the 1-methyl compound, dimerization occurs at the 4 position of the pyridine ring. While the evidence strongly favors this interpretation, the reservation may be held that the proof of structure is less than absolute. However, because this substance is referred to repeatedly below, a convenient designation is necessary and it will be called the "dimer." On the second wave, a mixture of DPNH and "dimer" is formed, and the "dimer" is very slowly reducible to

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† Eastman Kodak Fellow, 1964–1965.

[1] Abbreviations used in this work: DPN, diphosphopyridine nucleotide; DPNH, reduced DPN; sce, saturated calomel electrode; TPN, triphosphopyridine nucleotide; TPNH, reduced TPN.

DPNH. Both DPNH and the "dimer" are oxidizable to DPN^+ at anodes of appropriate potential. On the basis of the polarographic and controlled potential coulometric studies reported here, it is possible to outline basic reduction patterns for the nicotinamide derivatives including DPN, and to dispel much of the confusion in the literature, thereby laying the groundwork for the elaboration of detailed electrode reduction mechanisms by more sophisticated techniques.

Experimental Procedure

Materials. β-DPN^+ and β-DPNH were obtained from the Pabst Laboratories, Milwaukee, Wis., and yeast alcohol dehydrogenase from Worthington Biochemicals, Corp., Freehold, N. J. Coenzyme activities were assayed by published methods (Colowick and Kaplan, 1955). Tetra-*n*-butylammonium hydroxide and chloride of "polarographic grade" were obtained from Southwestern Analytical Chemicals Co., Austin, Texas. Deionized water was used throughout, and all other chemicals were of reagent grade.

Apparatus. The apparatus for polarography and controlled potential coulometry was the same as described previously (Burnett and Underwood, 1965). Polarograms were recorded at 25 ± 0.05° by means of a constant temperature water bath. The dropping mercury electrode had the following characteristics with a mercury head of 54 cm: m = 1.866 mg/sec; t = 4.6 sec/drop. This electrode was replaced by a conventional rotated platinum microelectrode in studying the anodic wave of DPNH. In all macroelectrolysis experiments, the electrolysis vessel was immersed in an ice–water bath. An atmosphere of nitrogen was maintained at all times. Absorption spectra were obtained with a Cary Model 14 spectrophotometer, and individual absorbance measurements were performed with a Beckman Model DU spectrophotometer. pH values were measured with a Beckman Zeromatic pH meter with glass and calomel electrodes. In some of the electrolysis experiments ultrasonic agitation was obtained by placing the cell in ice water contained in the tank of a Di Son Tegrator, System 40, made by Ultrasonic Industries Inc., Long Island, N. Y.

Polarography. Solutions of DPN^+ ranged from 1×10^{-5} to 1×10^{-3} M. Additional supporting electrolyte was not required at the buffer concentrations employed except in those experiments where the ionic strength was under investigation. Either gelatin or the cationic surfactant Hyamine 1622 was present as a maximum suppressor at a level of 0.005%. The tetra-*n*-butylammonium carbonate buffer was prepared by bubbling carbon dioxide through a 0.1 M solution of tetra-*n*-butylammonium hydroxide until the desired pH was obtained. In experiments where this buffer was employed, 1 M tetra-*n*-butylammonium chloride was substituted for the usual saturated potassium chloride in the reference electrode, but all potentials in this paper are referred to the saturated calomel electrode (sce).

Macroelectrolysis Experiments. In most of the electrolysis experiments, 90–700 mg of the electroactive species was present in 200 ml of 0.1 M buffer of pH 7–9 which was 0.1 M in potassium chloride. In some cases the solutions contained 0.0005% of Hyamine 1622. Vigorous ultrasonic agitation was employed in several of the experiments. Careful blank corrections were made by pre-electrolyzing the buffer solution before the DPN^+ was added, and then carrying the electrolysis to the same background current value. Polarograms were recorded just before and immediately after the electrolysis, as were ultraviolet spectra. Solutions were assayed using yeast alcohol dehydrogenase before and after each electrolysis.

For macroelectrolyses on the first reduction wave of DPN^+, the cathode potential was −1.2 v *vs.* sce. For electrolyses on the second wave, the cathode potential was −1.85 v. Reduction of the "dimer" to DPNH was studied by electrolyzing at −1.85 v solutions which had been subjected to complete prior electrolysis at −1.2 v. The potential employed for reoxidation of the "dimer" to DPN^+ was such that the potentiostat was not required; this process was accomplished by simple "internal electrolysis," *i.e.*, the mercury electrode (now an anode) was simply shorted to a large silver–silver chloride electrode through the input resistor of the coulometer, and the cell was allowed to run down. The oxidation of DPNH to DPN^+ required a more positive potential than could be attained with a mercury anode. In these experiments, the working electrode was an ordinary platinum gauze electrode of the type employed as an anode in large-scale analytical electrodeposition. Commercial DPNH rather than electrolytically reduced material was employed in studying this oxidation.

Results

Polarography. Many of our observations of the first DPN^+ reduction wave are in substantial agreement with those of previous workers (Kaye and Stonehill, 1952; Carruthers and Tech, 1955; Ke, 1956a) and accordingly need not be presented in detail. As before, over pH range 4–9 in acetate, citrate, phosphate, and pyrophosphate buffers, the half-wave potential was about −0.93 v *vs.* sce and was independent of the pH. When tested in a phosphate buffer of pH 8 over a DPN^+ concentration range of 1×10^{-4} to 1×10^{-3} M, i_d/c values were constant at 2.22 μa/mM. The height of the wave varied directly with the square root of the corrected mercury height, indicating that the limiting current for the reduction process was diffusion controlled. The plot of E *vs.* $\log (i_d - i)/i$ exhibited the slope expected of a reversible, one-electron reduction. In tetra-*n*-butylammonium carbonate buffers, the half-wave potential was somewhat more negative, about −1.12 v *vs.* sce. With the more dilute DPN^+ solutions ($<10^{-4}$ M) and thus with the sensitivity of the polarograph correspondingly high, a small concentration-independent prewave was seen at about −0.90 v. Although the prewave has not been studied in detail, it may be noted that the usual interpretation of such a wave in-

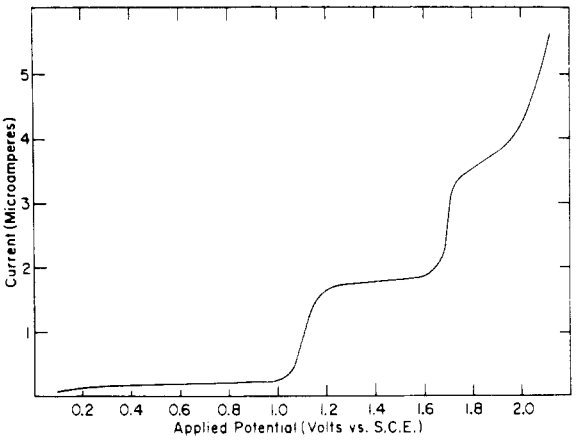

FIGURE 1: Polarogram of DPN solution prior to electrolytic reduction experiment. Conditions: 6.14×10^{-4} M DPN; 0.1 M tetra-*n*-butylammonium carbonate buffer, pH 9.1; 0.1 M potassium chloride.

vokes adsorption on the mercury surface. Such a phenomenon is better studied by techniques other than polarography, such as chronopotentiometry and double-layer capacitance measurements.

The second reduction wave of DPN^+ is reliably observed only under carefully specified conditions, and it is not surprising that it was overlooked by earlier investigators. For example, in phosphate buffers of pH 9 the wave was sometimes barely discernible as an ill-defined shoulder on the steeply rising current representing breakdown of the background electrolyte. However, in tetra-*n*-butylammonium carbonate buffers, a well-formed wave was easily observed. Figure 1 shows a typical DPN^+ polarogram in such a solution. The height of the second wave was proportional to about the one-third power of the corrected mercury height, intermediate between the values for diffusion control (one-half power) and for kinetic control (zero power). Slopes of E *vs.* log $(i_d - i)/i$ plots varied from about 0.025 to 0.1, indicating that the electrode process is an irreversible one. Values of α, the transfer coefficient, varied from about 0.44 to 0.54 when calculated assuming a two-electron process.

Even under the most favorable conditions which we were able to devise, the reproducibility of wave heights and half-wave potentials was rather poor. The scatter in $E_{1/2}$ values (standard deviation about 20 mv), the restricted pH regions of carbonate buffer effectiveness, and the instability of DPN^+ at extreme pH values prevented a completely satisfying study of the pH dependence of the second wave. However, within experimental error, over the pH range of 6 to 9, $E_{1/2}$ was independent of pH. This may be contrasted with the behavior of the 1-methyl model compound, which exhibited nearly the expected pH dependence for a two-electron process involving one hydrogen ion (Burnett and Underwood, 1965). The dependence of $E_{1/2}$ upon ionic strength was nearly buried within the experimental error, but there appeared to be a trend in the negative direction with increasing μ (-40 to -50 mv in going from $\mu = 0.01$ to 0.10). The mean of 15 values of $E_{1/2}$ in the pH range 7–9 at $\mu = 0.1$ was -1.72 ± 0.01 v *vs.* sce.

Macroelectrolysis on the First Wave. At a potential on the first wave, reduction was complete in 1 to 3 hr depending upon the DPN^+ concentration. Experiments in both phosphate and carbonate buffers at pH values of 7, 8, and 9 yielded the same results. Coulometry showed an uptake of one electron/molecule of DPN^+ (the mean of eight results was 0.98 ± 0.09, where the uncertainty is expressed as a confidence interval of the mean at a probability level of 0.95). The electrolyzed solutions exhibited neither DPN^+ nor DPNH activity when assayed with yeast alcohol dehydrogenase. Ultraviolet spectra were practically identical with that of DPNH, showing the characteristic band at 340 mμ which is generally associated with the 1,4-dihydropyridine structure. These results are essentially the same as those of Ke (1956a,b) in all regards except one: he reported (1956a) an n value of two under conditions not much different from ours. The cause of this discrepancy cannot be definitely assigned, but it may be noted that Ke's coulometric method (direct millicoulometry under polarographic conditions) is often unreliable (Hume, 1956; Weaver and Whitnack, 1958; Cover and Meites, 1961). On the basis of the ultraviolet spectrum, the erroneous n value, a paper chromatographic R_F value, and inactivity as a coenzyme, Ke concluded (1956b) that the reduction product was "very similar but not identical with enzymatically prepared DPNH."

The best interpretation of the results is that, at a potential on the first wave, DPN^+ undergoes a one-electron reduction with the formation of free radicals which subsequently dimerize at the 4 position of the pyridine ring. It would be expected that a 4,4′ dimer would exhibit essentially the same ultraviolet spectrum as the corresponding 1,4-dihydro monomer, since it possesses the same chromophore. Inactivity of a dimer as a coenzyme for yeast alcohol dehydrogenase would not be a surprising case of enzyme specificity.

Reoxidation of the "Dimer" to DPN$^+$. After complete electrolysis of DPN^+ at -1.2 v, representing quantitative conversion to the substance believed to be a dimer, the solution exhibited an anodic polarographic wave with a half-wave potential of about -0.25 v. Thus the potential of a calomel or silver–silver chloride electrode was sufficiently positive to accomplish the oxidation, and an external voltage was not required. Coulometry during such an internal electrolysis showed that one electron was lost for each DPN^+ molecule (measured n value: 0.91), and an enzymatic assay on the reoxidized solution gave a 95.3% recovery of the original DPN^+.

Reduction of the "Dimer" to DPNH. The dimer obtained from 1-methyl-3-carbamidopyridinium chloride was not reducible electrolytically to the 1,4-dihydropyridine (Burnett and Underwood, 1965). DPN^+ is different in this regard. Solutions of the "dimer" pre-

pared electrolytically exhibited a *very* small (scarcely measurable) wave at about −1.7 v. When such solutions were electrolyzed at −1.85 v, DPNH activity very slowly developed, amounting to about 10% conversion of the original DPN^+ after 10 hr. There was, of course, no change in ultraviolet spectrum.

Macroelectrolysis on the Second Wave. Electrolysis at a potential on the second wave is not clear-cut. Active DPNH is formed, but the rate of the electroreduction is very slow. In one experiment, for example, conversion of 88 mg of DPN^+ into DPNH was 38% complete in 3 hr; after 54 hr the value was still only 42%. This is in marked contrast to the complete reduction of the 1-methyl model compound to the 1,4-dihydro product in 2 to 3 hr. Addition of a cationic surfactant increased the rate appreciably, and it was considerably increased by vigorous ultrasonic agitation of the mercury–solution interface. For example, in one experiment the reduction of 96 mg of DPN^+ to DPNH was 59% complete in 2 hr. Coulometric n value measurements were very imprecise in these experiments chiefly because of the large background current at the very negative potential employed, but values between 1 and 2 were obtained which correlated, within experimental error, with the enzymatic DPNH assays.

The ultraviolet spectra of solutions which had been electrolyzed for long periods showed that, although conversion to DPNH was far from complete as shown by the enzymatic assay, all of the DPN^+ had been transformed into material with the 340 mμ chromophore. This was confirmed by assaying the solutions enzymatically for DPN^+ activity, which was invariably absent. Further, such solutions exhibited the anodic polarographic wave which is characteristic of the "dimer." Thus it is clear that both "dimer" and DPNH are obtained by electrolysis on the second wave. From the studies of the electrolytic reduction of the "dimer" itself, it appears that the rate of conversion of "dimer" into DPNH is far too slow to account for the rate of DPNH formation observed here. Thus it is seen that "dimer' formation and DPNH formation compete in the early stages of the electrolysis on the second wave while the very slow development of DPNH activity in the later stages is due to reduction of the "dimer." In one experiment, the solution after electrolysis at −1.85 v was subjected to the internal electrolysis mentioned above to reoxidize the "dimer" to DPN^+ (DPNH is not reoxidized at this potential; see below). Portions of the solution were then assayed for DPN^+ and DPNH. The two assays accounted for about 80% of the initial DPN^+. Failure of these assays to add up to 100% must be viewed in the light of the combined errors of three different enzymatic analyses and the fact that the experiment was performed over a period of more than two days, during which there may well have occurred some decomposition of the coenzyme solution.

Electrolytic Oxidation of DPNH. A sample of DPNH (108 mg) was electrolyzed in a phosphate buffer of pH 6 at a platinum working anode controlled at +1.05 *vs.* sce, polarograms with a rotating platinum microelectrode having shown a rather poorly defined wave in this region. A poor coulometric n value of about 2.4 was obtained, probably because of some evolution of oxygen at the platinum anode. The resulting solution, by enzymatic assay for DPN^+, showed a quantitative recovery.

Discussion

With 1-methyl-3-carbamidopyridinium chloride two polarographic waves are readily observed in various buffers, whereas with DPN^+ the second wave is often obscured in the breakdown of the background electrolyte. This is true despite the fact that the $E_{1/2}$ values for the second wave are about the same for both compounds. Thus it appears that some component of the DPN^+ system lowers the hydrogen overvoltage or in some other way decreases the useful potential range of the mercury cathode. In any case, both DPN^+ waves are readily observed in tetra-*n*-butylammonium carbonate buffers, and it is significant that the polarographic behavior of DPN^+ is now found to be in general harmony with that of the simple nicotinamide model compounds.

As described above, the electrode process for the first DPN^+ wave is reversible according to the usual polarographic criteria, but the reduction product ultimately obtained by electrolysis on this wave is reoxidized only at a much more positive potential. From these facts one visualizes a reversible electron-transfer process followed by an irreversible chemical step. Charge transfer leading to free radicals which subsequently dimerize is frequently observed in organic polarography, and it is probable, in our view, that this is the case here. In summary, the evidence for the formation of a dimer at a potential on the first wave includes the following: polarography and coulometry indicate that the product is formed by a one-electron process followed by an irreversible chemical step; the product possesses neither DPN^+ nor DPNH activity with yeast alcohol dehydrogenase; its ultraviolet spectrum is exactly that expected for the chromophore of a 4,4′ dimer; the model compound 1-methylnicotinamide definitely yields a dimer during electrolysis on the first wave (Burnett and Underwood, 1965); electrolysis of 1-*n*-propylnicotinamide appears to yield a 4,4′ dimer according to the work of Paiss and Stein (1958); and finally, there is evidence that a DPN dimer was obtained by the X-irradiation of DPN^+ solutions in aqueous ethanol (Swallow, 1955; Stein and Swallow, 1958). Thus it appears that previous workers who attempted the electrolytic preparation of DPNH at a potential on the first wave in fact obtained the 4,4′ dimer of DPN^+.

Powning and Kratzing (1957) electrolyzed with a cathode potential of about −1.7 v *vs.* sce and reported that "at least half" of the DPN^+ could be reduced to DPNH. This working potential was very near $E_{1/2}$ for the second wave, and it is probable that, as in the present case, they obtained both "dimer" and DPNH. Kono (1957) at −1.75 v and Kono and Nakamura (1958) at −2.0 v *vs.* sce obtained the highest reported yields of DPNH (65–76%), somewhat higher than our best

results. They noted that non-DPNH material absorbing at 340 mμ was also formed; this was the "dimer" in the light of our results.

Kono (1957) noted that a fraction obtained from etiolated mung bean seedlings, unlike yeast alcohol, lactic acid, or malic acid dehydrogenases, was capable of eliminating completely the 340 mμ absorption band of the electrolyzed solution. In a later paper (Kono and Suekane, 1958), an ammonium sulfate protein fraction obtained from an extract of the homogenized seedlings was studied further, and an electrophoretic fraction of much higher specific activity was obtained. Kono and Suekane state: "The enzyme differs from the other enzyme (diaphorase) in two characters. The one is that the enzyme can completely oxidize both kinds of DPNH obtained by dithionite and electrolytic reduction, while the other diaphorases or dehydrogenases are not able to oxidize the latter completely. . . ." It is now clear that Kono's preparation was capable of oxidizing both DPNH and the DPN "dimer"; the product, as he showed, was DPN^+. It is extremely interesting that there occurs an enzyme capable of acting upon the DPN "dimer." The significance of this unfortunately escaped Kono because he had no way of knowing that the "dimer" was present in his electrolytic DPNH preparations. The enzyme was not found in extracts of spinach, turnip, yeast, pig liver, or pig heart muscle (Kono and Suekane, 1958).

The work reported here suggests many other experiments. Electrolyses performed with a special cell in the microwave cavity of an electron spin resonance spectrometer in water or other solvents might disclose the free radicals. Rate studies to determine on the second wave the fraction of DPN^+ going to DPNH *via* the "dimer" as compared with direct addition of a second electron to the radical might be instructive. Electrode mechanisms could be approached by current reversal chronopotentiometry, cyclical scan voltammetry, and other modern electrochemical techniques. TPN^+ should be studied to learn why the yield of TPNH is nearly quantitative (Powning and Kratzing, 1957; Kono and Nakamura, 1958), while an appreciable fraction of DPN^+ under the same conditions goes to the "dimer." Finally, the "dimer" should be prepared and isolated in sufficient quantity for its structure and properties to be studied thoroughly.

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