A POLAROGRAPHIC AND COULOMETRIC STUDY ON DIPHOSPHOPYRIDINE NUCLEOTIDE

by

BACON KE

Charles F. Kettering Foundation, Yellow Springs, Ohio (U.S.A.)

Diphosphopyridine nucleotide (DPN) and its reduction product constitute one of the most important cosubstrates in a large number of biological oxidation-reduction reactions. The oxidation-reduction potential has been the subject of study by various authors[1-5]. Early values of the oxidation-reduction potential of the DPN system were derived from the equilibrium and from the thermochemical data. The E_0' value for the DPN system at 30° C and pH 7 was estimated by BORSOOK[1] to be —0.28 V. A more recent calculation based on the data of RACKER[2,3] has yielded a value of —0.30 V. Difficulties in such determinations lie in the inaccuracy in determining the equilibrium constants and the uncertainty in the potential values of the substrate systems. More recently, careful studies were made by BURTON AND WILSON[4], and by RODKEY[5]. Their results essentially agree with each other. Calculating from the equilibrium data, BURTON AND WILSON arrived at the value of -0.325 ± 0.004 V at 30° and pH 7. RODKEY determined E_0' by direct potentiometry and his value was reported to be -0.318 ± 0.003 V at 30° and pH 7.

In the present work studies on the electrochemical behavior of the oxidized and reduced diphosphopyridine nucleotide at the dropping mercury electrode will be reported. Coulometric technique has been used to study the number of electrons involved in the oxidation-reduction process of the DPN system and in the quantitative electrolytic reduction of DPN at polarographically controlled potential.

EXPERIMENTAL

Materials

Oxidized DPN was obtained from Pabst Laboratories and was used without further purification. It was assayed 89.3 % by enzymic reduction with ethanol and alcohol dehydrogenase and determination of the absorption at 340 mμ[6]. Alcohol dehydrogenase was obtained from Nutritional Biochemicals. Tris buffer was prepared from tris(hydroxymethyl)aminomethane obtained from Sigma Chemical Co. ("Sigma 7-9 buffer"). HCl, KCN, $CdCl_2$, KH_2PO_4, citric acid were either reagent grade or C.P. grade and were used without further purification. Tetramethylammonium chloride was of white label grade manufactured by Eastman Kodak.

Apparatus

A Leeds and Northrup glass electrode pH-meter was used for pH and potential measurements. Polarographic measurements were made with a Sargent model XXI automatic recording polarograph. This instrument was also used as a power supply in millicoulometric studies and micro-scale controlled potential electrolysis, since its output potential was precisely known. Micro cells for coulometric and electrolysis studies were constructed according to DE VRIES AND KROON[7]. Large scale electrolysis was performed with manually controlled potential in Sargent electrolysis vessels with interchangeable half cells. A model D-612 filtered D.C. power supply made by Electro Products Laboratories, Chicago was used as the polarizing unit. Current was measured with a

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Simpson D.C. multiple range ammeter. The Leeds and Northrup pH meter was used as the potential controlling instrument when the applied potential was below 1.1 V. Voltages above 1.1 V were controlled with an RCA vacuum tube voltmeter.

Methods

Enzymic. Spectrophotometric measurements were made with a Beckman model DU spectrophotometer, with quartz cells of 1 cm light path. Both oxidized and reduced DPN were measured by enzymic assay with alcohol dehydrogenase. Tris buffer of pH 8.8[3] was used as the medium. 5 ml DPN stock solution (approximately 1 mg per ml tris buffer) was pipetted into a 10 ml volumetric flask, 0.02 ml alcohol dehydrogenase (0.4 mg protein) and 0.5 ml alcohol were then added. The increase in light absorption at 340 mμ was then taken after 30 min or when it reached a constant maximum value. A molar extinction coefficient of $6.22 \cdot 10^6$ cm^2 $mole^{-1}$ was used to make calculations.

Polarographic. Current-voltage curves were usually taken under an applied potential starting from 0 up to the potential at which hydrogen ion is reduced. An external saturated calomel electrode (S.C.E.) was used as the reference electrode for all polarographic measurements. All solutions to be polarographed were deaerated for 10–15 min with nitrogen freed of oxygen (same deaeration for all solutions used in coulometric analysis and controlled potential electrolysis). The dropping Hg electrode used here has a $m^{2/3} t^{1/6}$ value of 2.17 $mg^{2/3}$ $sec^{-1/2}$ in 0.1 *M* pH 7.65 tris buffer at the potential on the diffusion current plateau. All measurements were made at 25° C.

Coulometric. The millicoulometric technique of DE VRIES AND KROON[7] was used for the present studies. 0.3 ml DPN solution was placed in one cell and an equal amount of $CdCl_2$ solution was placed in a second cell connected in series with the first cell. A potential equal to $E_1 + E_2$ was applied across the cells in series for approximately 3 hours, where E_1 and E_2 are the potential values at the plateau of the two diffusion currents measured separately. Oxygen-free nitrogen was passed over the solution during the course of electrolysis. Ordinary marine barometer capillary tubing wrapped with an internal gauge 22 Ag-wire Ag-AgCl reference electrode[8] was found very satisfactory with tris buffer, since the latter contains chloride ions. 0.3 ml solution was a convenient amount for this procedure.

Electrolysis. Micro-scale electrolysis was carried out in the same type micro cells used in millicoulometry, with all procedures essentially the same except that the electrolysis was performed for a much longer period of time. Large scale electrolysis was performed in a H-cell according to procedures of LINGANE[9]. Mercury was used both as cathode (the working electrode) and anode. At the anode, Hg was oxidized to the oxide. Either a saturated calomel electrode linked through an agar-bridge or an Ag-AgCl electrode[10] may be used as the reference electrode. Deaerated DPN solution in 0.1 *M*, pH 7 tris buffer was used as the catholyte; the same buffer without DPN was placed in the anode compartment. A glass stirrer was mounted near the solution-mercury interface.

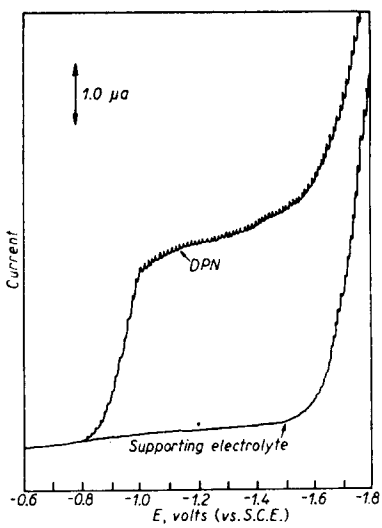

Fig. 1. Polarographic waves for tris buffer and DPN in tris buffer.

RESULTS AND DISCUSSION

Polarographic studies

0.1 *M* tris buffer solutions in the neutral pH region do not yield any polarographic waves over a wide range of applied potentials. A dilute solution of DPN (*e.g.* $1.35 \cdot 10^{-3}$ *M*) in 0.1 *M*, pH 7.65 tris buffer gave a well defined polarographic wave with $E_{1/2}$ equal to —0.936 V (*vs.* S.C.E.) (Fig. 1).

When a series of buffer solutions containing known but different amounts of DPN were polarographed, the current-voltage curves showed that the diffusion current was directly proportional to the concentration of the electroactive material (DPN). The values of the diffusion current constant ($I = i_d/Cm^{2/3} t^{1/6}$) are listed in Table I. The individual I values agree within 1%. This I value should be useful in computing the concentration in an unknown case from the observed value of i_d and the measured

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values of m and t for the particular electrode used. Furthermore, the diffusion coefficient of DPN may also be evaluated from the diffusion current constant by means of the Ilkovic equation. Thus $D = (i_d/607\, n\, C\, m^{2/3} t^{1/6})^2 = (I/607\, n)^2 = 1.46 \cdot 10^{-6}$ $cm^2\, sec^{-1}$. Although the theoretical basis for such calculation is not yet sufficiently established, the resulting value may be considered as approximately true.

TABLE I

DIFFUSION CURRENT CONSTANT FOR DPN

DNP concn. (mM)	i_d *(μa)*	I $(i_d/Cm^{2/3}t^{1/6})$
6.048	19.6	1.49
3.024	9.6	1.47
1.512	4.9	1.49
0.756	2.4	1.46
0.378	1.2	1.44

Mean value 1.47

When $-E_{d.e.}$ was plotted against log i/i_d—i for the current-voltage curve of DPN in tris buffer a straight line with a slope of 0.08 was obtained. However, this plot was of no use in calculating the number of electrons involved in the reduction of DPN since the latter reaction was demonstrated to be irreversible at the dropping mercury electrode (see below).

Another evidence for the irreversibility of the DPN reduction at the dropping Hg electrode is the independency of its half-wave potentials from the pH of the supporting electrolyte at values below 7. Between pH 7 and 9 there is a slight shift of the half-wave potentials toward more negative values with increasing pH (Table II).

TABLE II

EFFECT OF THE NATURE AND THE pH OF THE SUPPORTING ELECTROLYTE ON THE HALF-WAVE POTENTIAL

Supporting electrolyte	*Concn. (M)*	*pH*	$-E_{1/2}$ *V (S.C.E.)*
Phosphate-citrate	0.1	3.20	0.92
Phosphate-citrate		4.30	0.92
Phosphate-citrate	0.1	5.23	0.91
Phosphate-citrate	0.1	6.20	0.91
Phosphate-citrate	0.1	7.10	0.92
Tris	0.1	7.25	0.92
Tris	0.1	7.44	0.93
Tris	0.1	7.57	0.92
Tris	0.1	7.90	0.93
Tris	0.1	8.09	0.93
Tris	0.1	8.28	0.94
Tris	0.1	8.53	0.93
Tris	0.1	8.68	0.94
Tris	0.1	8.97	0.95
Tris	0.1	9.24	0.96
Tetramethyl ammonium chloride	0.3	4.96	0.97 1.38
Tris (0.1 *M* in KCN)	0.1	0.95	—

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A numerical value of 3 was derived for n from the $E_{1/2}$ *vs.* pH plot if reversibility was assumed for the electrode reaction.

It should be noted that the half-wave potential of DPN in the phosphate-citrate buffer solutions is nearly the same as in the tris buffer. When 0.3*M* aq. solution of tetramethylammonium chloride was used as the supporting electrolyte for DPN, the polarogram obtained showed two separate waves of nearly equal wave heights with half-wave potentials at −0.97 and −1.38 V (*vs.* S.C.E.) respectively. The waves are considerably more drawn out than in the buffered solutions. When the current-voltage curve was taken for DPN in tris buffer containing 0.1*M* KCN, no polarographic wave occurred either at −0.94 V or at more negative potentials. It is well known[11] that DPN forms with cyanide an addition compound DPN–CN. The DPN–CN complex resembles reduced DPN in its absorption spectrum and certain other chemical properties. The present investigation indicates that this addition compound is not reducible at the dropping mercury electrode.

If the reduction of DPN at the dropping Hg electrode were a reversible reaction, the half-wave potential would have assumed the same value as the oxidation-reduction potential of the DPN system. The deviation of the half-wave potential of DPN from the oxidation-reduction potential represents the cathodic overvoltage at the current density corresponding to $i_d/2$. The existence of such an overvoltage may also be used as the most conclusive evidence for the irreversibility of the electrode reaction. The DPN system belongs to the so-called "sluggish systems", which require long periods of time to establish significant potential at the indicator electrode. Hence, in the potentiometry a "mediator" of suitable normal potential has to be added to the DPN system to "poise" the electrode potential. While the cathodic processes involve negative overvoltages, the overvoltages for the anodic processes are positive (see Fig. 2).

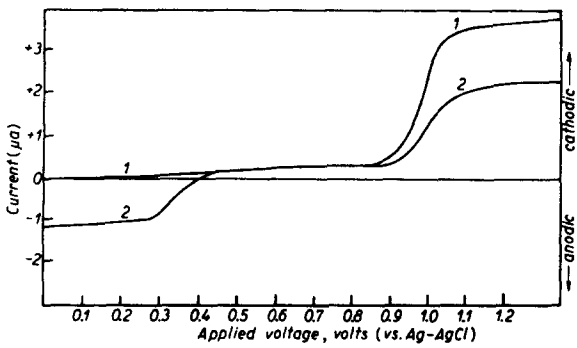

Fig. 2. Composite cathodic-anodic wave for the DPN system. Curve 1 obtained before electrolysis. Curve 2 obtained after 125 min electrolysis.

Coulometric measurements and electrolysis

Attempts were made to calculate the number of electrons involved in the reduction of DPN, since this is one of the most important quantities for the elucidation of the reaction mechanism. In the present case, however, this was not possible either from the diffusion current data or from the pH *vs.* $E_{1/2}$ plot, since the thermodynamic reversibility of the wave could not be established. Consequently, independent determinations of n by means of direct coulometric measurements had to be made.

The millicoulometric technique used here is based on the principle that for a certain amount of current passed through two cells in series, there is an equivalent amount of electrochemical change occurring in each cell. The change in the concentration can be determined by measuring the decrease in wave height. If one cell contains a compound for which the number of electron change is known, a comparison

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of the concentration changes in the two cells will lead to the number of electrons involved in the reaction of the substance in question.

The data obtained from millicoulometric measurements may be substituted into the following equation to calculate the number of moles of the compound that is reduced:

$$\Delta N = \left[1 - \frac{i_2}{i_1}\left(\frac{t_1}{t_2}\right)^{1/6}\right] N$$

where ΔN is the amount of material which is reduced, N is the number of moles of the compound in the cell before electrolysis, i_1 and i_2 are the diffusion currents at the beginning and end of a run, t_1 and t_2 are the corresponding drop times. Typical results are listed in Table III.

TABLE III

COULOMETRIC ANALYSIS ON DPN

Expt. No.	*Compound*	*Concn. (mM)*	i_d *(μa)*		*t (sec)*		ΔN *(mM)*
			initial	*final*	*initial*	*final*	
1	$CdCl_2$*	1.68	11.10	9.60	3.90	3.63	0.212
	DPN	0.82	3.16	2.36	3.90	3.72	0.208
2	$CdCl_2$*	1.63	12.56	8.16	3.20	3.15	0.571
	DPN	1.09	3.22	1.68	3.10	3.09	0.522

* $CdCl_2$ in 0.1 *M* KCl.

These ΔN values gave ratios of approximately unity. Hence it may be concluded that $n = 2$ which is reasonable for DPN reduction on the basis of other experimental evidence. Thus the electro-reduction may be represented by the commonly accepted equation:

$$DPN^+ + 2H^+ + 2e \longrightarrow DPNH + H^+$$

In another series of experiments, single micro cells containing 0.3 ml of approximately 1 millimolar DPN solutions were used for simultaneous polarographic and electrolysis studies. A polarogram was taken before electrolysis, then the solution was subject to electrolysis at a controlled potential for several hours until an appreciable fraction of the oxidized DPN was converted to the reduced form. A polarogram taken after 125 min electrolysis showed a composite wave consisting of an anodic wave due to the oxidation of DPNH and the cathodic wave (Fig. 2). Fig. 3 shows a study of the changes of the cathodic and anodic diffusion currents with time.

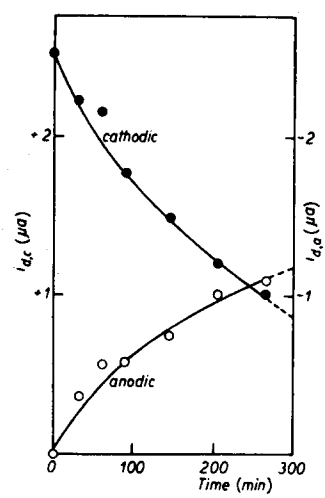

Fig. 3. Changes in the cathodic and anodic diffusion currents during the course of electrolysis.

Preliminary experiments on large scale electrolysis using a H-cell and 50 ml DPN solution were conducted at a controlled potential according to the procedure of LINGANE[9]. Small aliquots were removed from time to

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time, and the optical density readings were taken at 340 mμ. The readings showed a steady increase with time. At the end of electrolysis the absorption spectrum was also measured between 300 and 400 mμ and it possessed the characteristic shape of the reduced DPN. The final reading in optical density at 340 mμ indicated that the spectrophotometrically measurable amount of reduced DPN corresponds to 70% of the total DPN reduced by enzyme*. Further studies of this technique coupled with the application of automatic instrumentation (*e.g.* potentiostat) should develop a convenient and precise preparative method for reduced DPN.

At the writing of this paper, an article by CARRUTHERS AND TECH on the polarography of the di- and triphosphopyridine nucleotides appeared in the literature[12]. According to these authors TPN has a half-wave potential of −1.23 V (*vs.* S.C.E.) in a 0.5*M*, pH 10.3 to 10.6 tris buffer, and based on these polarographic properties an analytical method was devised for the quantitative determination of DPN and TPN in the extracts of living tissues, especially mouse liver.

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SUMMARY

The electrochemical behavior of diphosphopyridine nucleotide was studied at the dropping Hg electrode. The half-wave potentials and the diffusion current constant were measured, and the effect of the pH and the nature of the supporting electrolyte on the current-voltage curves were also studied. The irreversibility of the electrode reaction was established. Overvoltages were observed for the cathodic and anodic waves.

The number of electrons involved in the electrode reaction was determined by direct coulometry. Controlled potential electrolysis was suggested as a preparative method for reduced DPN.

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