[CONTRIBUTION FROM THE CHARLES F. KETTERING FOUNDATION, YELLOW SPRINGS]

Electrolytic Reduction of Diphosphopyridine Nucleotide at Some Solid Metal Electrodes

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The electrolytic reduction of DPN at some solid metal electrodes is described. The polarization curves and the data on the coenzyme activity of the reduction product also are reported. Some currently proposed mechanisms for the various modes of DPN reduction are discussed.

Diphosphopyridine nucleotide (DPN) has been found to be electrochemically reducible at the dropping mercury electrode and give a well-defined current–voltage curve with a half-wave potential of −0.936 v. (*vs.* S. C. E.).[1] The reduction product possesses a characteristic absorption band at 340 mμ and some other properties similar to those of the chemically or enzymatically reduced DPNH. However, it does not possess fluorescence and is not reoxidizable by acetaldehyde using alcohol dehydrogenase (ADH) as the catalyst.[2] Several other workers have also prepared totally inactive or partially active reduced DPN by using agents other than ethanol plus ADH or sodium hydrosulfite. Mathews and Conn,[3] using sodium borohydride as the reducing agent, have obtained a DPN reduction product which can be reoxidized enzymatically to the extent of about 50%. Swallow[4] reported the reduction of DPN by irradiating an air-free aqueous solution of DPN (3×10^{-4} *M*, *p*H 7.8) in the presence of 0.5 *M* ethanol. However, the reduction product was found totally inactive enzymatically. In the present communication the electrolytic reduction of DPN at some solid metal electrodes and the corresponding polarization curves together with the data on the coenzyme activity of the reduction product will be reported. Some currently proposed mechanisms concerning the various modes of DPN reduction will also be discussed.

Experimental

Materials.—DPN was a 90% pure preparation obtained from Pabst Laboratories. Tris buffer was prepared from tris-(hydroxymethyl)-aminomethane obtained from Sigma Chemical Co. ("Sigma 7-9 buffer"). Yeast alcohol dehydrogenase was a 2X crystalline preparation from Sigma. Metal electrodes and their sources are as follows: platinum and palladium sheets (American Platinum Works), lead (1/32 in.) and nickel (B. and S. 22) sheets (Central Scientific Co.) and silver foil (A. D. Mackay, Inc., New York).

Apparatus and Methods.—The Lingane–Jones potentiostat[5] was constructed for carrying out electrolytic reduction at controlled potentials. The electrolysis cell (Fig. 1) was a modification of the design used by Delahay and co-workers[6] and was made of lucite. The metal sheet electrode was thoroughly cleaned first and then clamped at the cell bottom by means of screws. The working area of the cathode (E_1) was 7.1 cm.^{2}. The glass stirrer was rotated by a Sargent synchronous rotator at 600 r.p.m. to ensure uniform circulation of the electrolyte. The saturated calomel electrode (S. C. E.) was used as the reference electrode (E_3). The tip of the S. C. E. is a capillary of a diameter of 0.9 mm. and is cut at an angle of 30 degrees. Such a reference

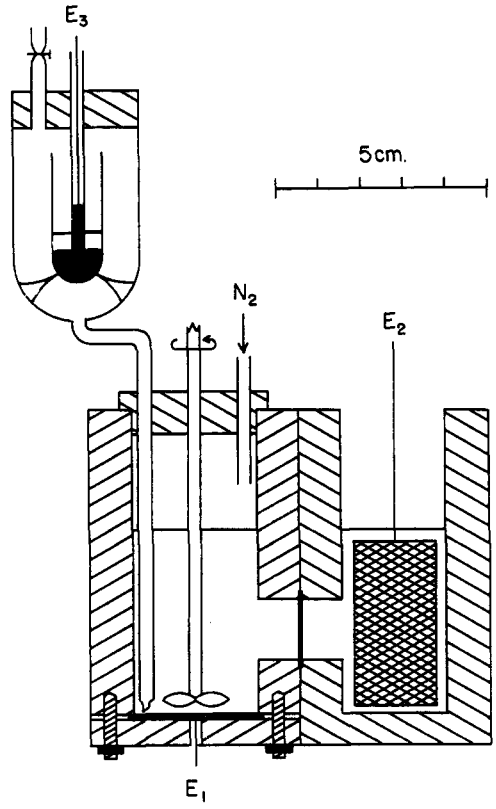

Fig. 1.—Electrolysis cell: E_1, working electrode; E_2, auxiliary electrode; E_3, saturated calomel electrode.

electrode introduces a negligible ohmic drop in the voltage between the working and the reference electrodes.[7] A Sargent–Slomin platinum gauze anode (22 mm. diam., 40 mm. high) was used as the auxiliary electrode (E_2) in all experiments. The anode compartment was clamped to the cathode compartment by means of screws and was separated by a piece of 0.0023 inch thick dialyzer membrane. This membrane is convenient to use and produces very little resistance in the electrolysis circuit.

Stock solution, 1.23×10^{-3} *M* DPN was made up with 0.2 *M* *p*H 7.5 Tris buffer and used in all electrolysis experiments. Twenty ml. of solution was sufficient for each run. The solution was deaerated with purified nitrogen for 10 min. immediately before electrolysis. Purified nitrogen was also passed through the cathode compartment at the rate of 2 bubbles per sec. to maintain a strictly inert atmosphere during the course of electrolysis. Tris buffer was used in the anode compartment.

Since both DPN reduction and H_2-evolution occur simultaneously at these solid metal electrodes at sufficiently negative potentials, the *c*–*v* curve of DPN reduction at these electrodes cannot be obtained directly from current and voltage measurements as at the dropping mercury electrode. Such a curve can be established, however, by the indirect method of Delahay[8] by electrolyzing the solution

(1) B. Ke, *Biochim. Biophys. Acta.*, in press.

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(3) M. D. Mathews and E. E. Conn, THIS JOURNAL, **75**, 5428 (1953).

(4) (a) A. J. Swallow, *Biochem. J.*, **54**, 253 (1953); (b) **61**, 197 (1955).

(5) J. J. Lingane and S. L. Jones, *Anal. Chem.*, **22**, 1169 (1950).

(6) P. Delahay, G. L. Stiehl, J. A. Perry and J. O. Juliano, Tech. Rep. O. N. R., Report 12, Project NR-051-258 (1954).

(7) P. Delahay, "New Instrumental Methods in Electrochemistry," Interscience Publishers, Inc., New York, N. Y., 1954, p. 392.

(8) Ref. 5, p. 295.

at constant potentials and following the amount of DPN reduced by the spectrophotometric method. The amount of DPN reduced was determined by measuring the light absorption at 340 mμ on a small volume of solution pipetted out from the cathode compartment at various time intervals. The enzymatic reoxidation of the electrolytically reduced compound was carried out with acetaldehyde using ADH as the catalyst.

Results

The change in DPN concentration during electrolysis at a given cathode potential is plotted *vs.* time in Fig. 2 for platinum electrode. Other metal electrodes show very similar relationships. The

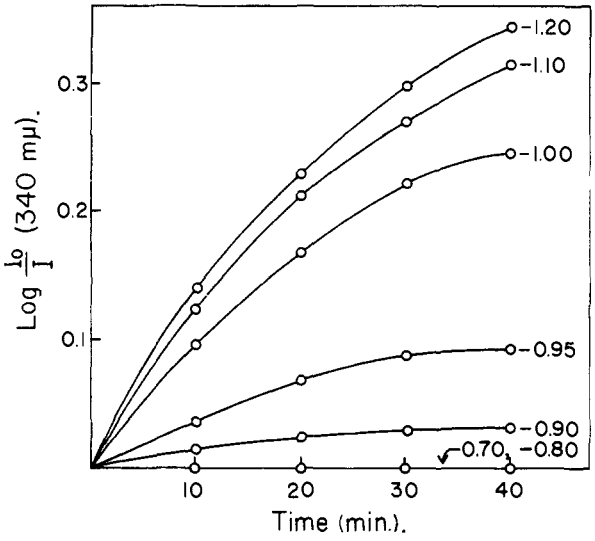

Fig. 2.—Change of DPN concentration with time during the electrolysis. The number designates the potential (*vs.* S. C. E.) of the working electrode.

optical density (D) of 0.5 ml. electrolyte diluted to 3.0 ml. was taken and plotted *vs.* time in minutes. dD/dt is the slope of the tangent to the curve D *vs.* t at time t. If dD/dt is taken at time $t = 0$, then current i corresponds to the initial concentration. Thus $i = nF(dN/dt) = nFV(dC/dt) = nFVd(D/\epsilon)/dt$, where dN/dt is the number of moles of DPN reduced per unit time at an electrode with an area of 7.1 cm.^{2}, i is the current, n is the number of electron changes during the reduction, F (= 96500 coulombs) is the faraday, C is the molar concentration of DPN, D is the optical density and ϵ the molar extinction coefficient which is taken as 6.22×10^3 cm.^{2} mole^{-1} (*vide infra*).

The complete current–potential curves for the various electrodes (Fig. 3) were established by determining the D *vs.* t plots for various potentials (Fig. 2). All polarization curves were measured from −0.7 to −1.2 v. *vs.* S. C. E. The shapes of the curves are practically identical to that of the polarogram obtained at the dropping mercury electrode.[1] The polarization curve for the silver electrode is shifted toward the negative side by approximately 25 mv., that for nickel electrode is shifted by 50 mv., while the other three are almost identical. The heights of the polarization curves differ slightly at −1.2 v. for the same initial DPN concentration and equal geometric area of the electrode surface. These differences may partially be accounted for by the surface structure of the metals

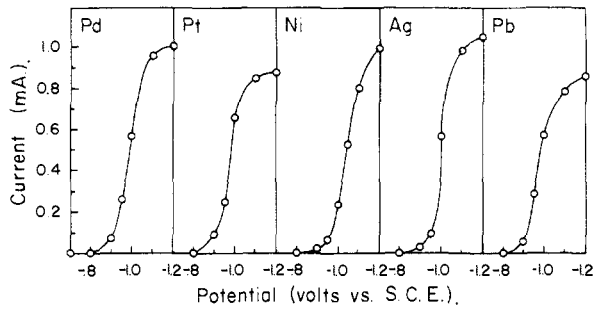

Fig. 3.—The current–potential curves for DPN reduction at various metal electrodes.

and they may be within the limit of error of the current measurements.

The *p*H of the catholyte remained practically constant during the electrolysis at most of the potentials applied. Even at −1.2 v. the *p*H usually increased by less than 0.2 unit after 40 min. electrolysis.

Contrary to the fact that the electrolytically reduced product formed on the mercury electrode possesses no coenzyme activity, the reduction compound at some of the solid metal electrodes shows partial activity. The results for the various electrodes are shown in Table I. The values listed represent an average of many determinations. $D_{initial}$ and D_{final} are the optical densities at 340 mμ of the electrolytically reduced product before and after enzymatic reoxidation respectively. The percentage of the enzymatically reoxidizable fraction in the total reduction product is listed in the last column. It is the ratio of the portion of optical density suppressed through enzymatic reoxidation to the $D_{initial}$ value. It should be pointed out, however, that since the molar extinction coefficient of the inactive reduction compound is considerably smaller than that of the enzymatically reduced DPNH[2], the % value calculated in this manner results in a higher value than it actually should be. For the same reason, the electric current derived from the 340 mμ absorption should actually be larger than that shown in Fig. 2.

One experiment was performed on the active portion of the reduction product obtained at the Pt electrode in which it was first reoxidized with acetaldehyde in the presence of ADH, and then reduced again enzymatically with excess alcohol. The conversion was quantitative when assayed by the spectrophotometric method.

Discussion

The results in Fig. 3 indicate that the current–potential relationships do not depend on the nature of the electrode materials. Since the electrode metals used here have different overvoltage values for hydrogen evolution, ranging from the lowest value in Pd to the highest in Hg, and that this difference in hydrogen overvoltages does not result in any variation of the current–potential curve positions, one may conclude that the reduction involves proton transfers rather than direct transfer of hydrogen atoms.

Cathodic reduction of organic molecules may proceed by way of two alternative types of mechanisms, *viz.*,

(A) Direct addition of hydrogen atoms

$$H^+ + e^- = MH$$
$$R + MH = RH + M$$

where R is the organic molecule and M is the electrode metal. Or

(B) Primary electron transfer followed by addition of protons

$$R + e^- = R^-$$
$$R^- + H_3O^+ \text{ (or } H_2O) = RH + H_2O \text{ (or } OH^-)$$

Hence the mechanism of the electrolytic reduction of DPN at the metal electrodes is controlled by the primary electron transfer as is shown by the constant current–potential relationships at the electrodes with variable rates of hydrogen evolution. Some direct evidence on DPN has been reported by Swallow[4b] who used the palladium foil diffusion apparatus to generate hydrogen atoms and did not observe any effect on the DPN solution.

It may also be noted that the results shown in Table I do not indicate any correlation between the percentage of the reoxidizable fraction of the reduction product and any of the intrinsic properties (hydrogen overvoltage or zero-charge potential, for instance) of the electrode materials. However, for the reason to be discussed below, an effect of the molecular orientation at the electrode surface during the electrode process should not be ruled out completely.

Recently Stein and Stiassny[9] have reviewed the various modes of DPN reduction and illustrated on a DPN model substance, nicotinamide-N^1-propyl iodide.

Burton and Kaplan[10] first proposed an ionic addition mechanism for the enzymatic and hydrosulfite reduction of DPN. This mechanism was supported by the works of Yamolinsky and Colowick,[11] and Swallow.[12] DPN reduction by the action of an irradiated system was first assumed by Stein and Swallow[13] to be due to the formation of free radicals. The mode of borohydride action is less certain, but it is often assumed to involve hydride ions. Working with nicotinamide-N^1-propyl iodide. Stein and Stiassny[9] have obtained characteristic absorption spectra for the reduction products formed by hydrosulfite and irradiation, respectively. On borohydride reduction of the nicotinamide, they obtained a product with a spectrum which is a composite of those of the reduction products formed by the other two methods. Furthermore, two pure components could be separated from this reduction product. They also reduced nicotinamide-N^1-propyl iodide electrolytically in an alkaline carbonate solution, and obtained a product the spectrum of which is identical with that of the radiation product and one of the borohydride products. The non-interference of the rate of hydrogen evolution on the electrode reaction of DPN observed in the present work tend to enhance the assumption that the electrolytic reduction proceed through a free radical intermediate.

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TABLE I

COENZYME ACTIVITY OF THE ELECTROLYTICALLY REDUCED DPN FORMED AT THE VARIOUS METAL ELECTRODES

D values were taken on 1 ml. of 1.23×10^{-3} *M* DPN electrolyzed for 60 min. at −1.4 v. (*vs.* S. C. E.) and diluted to 3.0 ml. final volume with 0.2 *M* *p*H 7.5 Tris buffer.

Electrode	$D_{initial}$	D_{final} (cor. for diln.)	Enzymatic reoxidation
Pd	0.760	0.760	0
Pt	.978	.613	37
Ni	.783	.787	0
Ag	.772	.773	0
Pb	.950	.422	56
Hg	...	...	0 (ref. 2)

The site of hydrogen transfer in the enzymatic and hydrosulfite reduction of DPN have recently been demonstrated by Pullman, San Pietro and Colowick[14] and by Loewus, Vennesland and Harris[15] with two different methods to occur at the *para* position of the pyridine ring, and it was also confirmed that only this reduction product would be enzymatically active. Hence it led Mathews and Conn,[3] and Stein and Swallow,[13] to suggest that in the DPN reduction by borohydride or irradiation an *ortho*-dihydro isomer might be formed. This may also be true in the case of electrolytic reduction. However, there is no direct experimental evidence for this speculation. Work is in progress in this Laboratory and we hope to gain more information on this point.

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