593. *The Mechanism of the Reduction of Some Nicotinamide Derivatives.*

By YEHUDA PAISS and GABRIEL STEIN.

The electrolytic reduction of *N*-propylnicotinamide, proceeding by a mechanism involving one-electron transfer and free-radical intermediates, results in the formation of a dimer and a dihydro-compound. This dihydro-derivative differs from that produced by reduction with dithionite, which involves addition of a negative ion and two-electron transfer. Sodium borohydride yields a mixture of the two dihydro-derivatives, either of which may then be isolated at will.

N-SUBSTITUTED nicotinamide derivatives are in many respects suitable models for the study of the mechanism of reduction of DPN^+ (coenzyme-I). They were studied by Karrer *et al.*,[1] who showed that reduction of nicotinamide propiodide by sodium dithionite gives a dihydro-compound which resembles DPNH in its properties. Colowick [2] and Swallow [3] have shown that the reduction is the result of a one-step, two-electron-equivalent transfer due to addition of a negative ion. Reduction of the coenzyme by dithionite yields an enzymically active product. Enzymic reduction involves transfer of a hydride ion.[4]

When *N*-substituted nicotinamides are reduced by free radicals, produced by irradiating the solutions with *X*- or γ-rays,[5] the product, a dihydro-compound, is not identical with the product of dithionite reduction. It was also shown that reduction by sodium borohydride [6] yielded a mixture of the two dihydro-compounds, which could be separated. Electrolytic reduction [6] produced the dihydronicotinamide also obtained from the irradiated solution. Similar results, in the case of borohydride, were obtained by Wallenfels and Schuly.[7] However the results were questioned by Karrer and his co-workers [8] who obtained by borohydride reduction only one dihydro-derivative, that resulting from dithionite reduction.

In the present paper it is shown that the absence of the other compound in Karrer's work is due to the experimental conditions employed. Conditions are now described which enable one to obtain either of the products, using borohydride. By the electrolyte method a dimer can also be isolated, in agreement with the postulated one-electron-transfer mechanism.

EXPERIMENTAL

Materials.—Sodium borohydride was obtained from B.D.H. Ltd., sodium dithionite from J. T. Baker Co. Nicotinamide methiodide, propiodide, and benzylochloride were prepared by the methods of Karrer's school.[1,8]

Spectra.—Ultraviolet spectra (see Figs.) were taken on a Beckman DU instrument. Fluorescence spectra were obtained on the same instrument fitted with the Beckman Special Fluorescence attachment, and Spectral Energy Recording Attachment. Infrared spectra were taken on a Baird double-beam instrument, in KBr discs.

Oxidative Titrations.—The solution was adjusted to pH 8—9 with borate buffer, and excess of 10^{-2}N-potassium ferricyanide solution in borate buffer (pH 8) was added. After 30 min.

[1] (*a*) Karrer, Schwarzenbach, Benz, and Solmssen, *Helv. Chim. Acta*, 1936, **19**, 811; (*b*) Karrer and Stare, *ibid.*, 1937, **20**, 418.
[2] Colowick, in "Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, 1954, p. 353; Yarmolinsky and Colowick, *Biochem. Biophys. Acta*, 1956, **20**, 177.
[3] Swallow, *Biochem. J.*, 1955, **60**, 443.
[4] Vennesland and Westheimer, in "Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, 1954, p. 357; Mahler and Douglas, *J. Amer. Chem. Soc.*, 1957, **79**, 1159.
[5] Stein and Swallow, *Nature*, 1954, **173**, 937; Stein, *J. Chim. phys.*, 1955, **52**, 634.
[6] Stein and Stiassny, *Nature*, 1955, **176**, 734.
[7] Wallenfels and Schuly, *Angew. Chem.*, 1955, **67**, 517.
[8] Brook, Blumer, Krishna, Schnell, and Karrer, *Helv. Chim. Acta*, 1956, **39**, 667.

2 ml. of 10% potassium iodide solution were added, followed by 2 ml. of 10% zinc sulphate solution and 3 ml. of 5N-hydrochloric acid. The iodide liberated was titrated with 10^{-2}N-thiosulphate. The same procedure was always carried out at the same time on a control solution.

Reduction by Sodium Dithionite.—This was carried out essentially according to the procedure of Karrer *et al.*[1] The propiodide (1 g.) was dissolved in M-sodium carbonate (20 ml.) at 0°. Whilst nitrogen was passed through the reaction mixture, small portions of solid dithionite were added. An orange colour appeared, fading to yellow. When further addition caused no further orange coloration the solution was extracted with peroxide-free ether (5 × 50 ml.). The ether extract was dried (Na_2SO_4) and distilled in the cold in vacuum until turbidity appeared. Addition of light petroleum (b. p. 40—60°, 5 ml.) precipitated crystals. Recrystallised from ether–light petroleum these had m. p. 92° (Found: C, 65·0; H, 8·4. Calc. for $C_9H_{14}ON_2$: C, 65·1; H, 8·4%). Oxidative titrations showed a mean value of 95% of the calculated. The absorption spectrum for an ether solution showed one maximum only, at 346 mμ (ε 5800). For aqueous carbonate solution the maximum is at 360 mμ. On addition of acid this peak disappears and a single new peak at 295 mμ appears. The maxima of the fluorescence spectrum lie at 430 mμ for ether and 480 mμ for 0·1M-sodium carbonate solution. We denote this product as dihydro-compound A.

Reduction by Sodium Borohydride.—The same procedure was used as with dithionite. The ether extract of the reaction mixture was dried (Na_2SO_4) and evaporated in a vacuum in the cold until turbidity appeared. The closed vessel was kept at −5°, till crystals were deposited; these had m. p. 103°, unchanged by recrystallisation. For ether solutions the spectrum

FIG. 1. *Absorption spectrum of dihydro-compound A (dashes) and B (solid curve).*

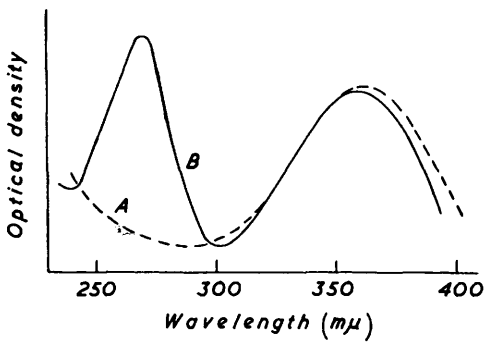
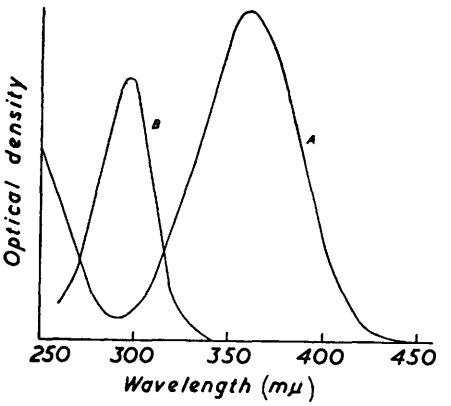


FIG. 2. *Absorption spectrum of dimer obtained by electrolytic reduction of nicotinamide propiodide* (3 × 10^{-2}M) in 0·1M-Na_2CO_3.



A, at pH 9. *B, at* pH 5.

(Fig. 1) has two peaks, at 265 and 350 mμ. At 350 mμ (ε 6700). The fluorescence spectrum shows one peak only at 443 mμ for ether and at 505 mμ for 0·1M-sodium carbonate solution. Oxidative titrations showed 95—98% of the theoretical value calculated for a dihydro-derivative. In the ether extract, after deposition of the crystals the spectrum exhibits both peaks, that at 350 mμ being the higher. If the solution of the new dihydro-derivative (B) in carbonate is acidified, both absorption peaks disappear and a single peak at 295 mμ appears, as with the product A.

For the preparation of B small quantities of nicotinamide propiodide and sodium borohydride were added alternately to N-sodium carbonate (3 ml.) at 0°. Borohydride was added after each addition of the substrate until no more orange colour was formed. Altogether 2 g. of starting material were used. Towards the end of the reaction the crystalline derivative B was precipitated. Recrystallised from warm water it had m. p. 103° (yield approx. 50%) (Found: C, 65·1; H, 8·25%).

Effect of Added Anions.—A few qualitative experiments were carried out on the rôle of the valency of added anions. If instead of sodium carbonate being used, the reaction solution was

adjusted to pH 11 by M-boric acid–sodium hydroxide buffer (H_3BO_3 being assumed effectively univalent at this pH), no crystalline product B was obtained, but an oil, which showed a spectrum indicating that it contained mainly A. If 0·1N-sodium carbonate was used, no crystalline product B was precipitated, but if 0·9N-sodium sulphate was also present, crystalline product B was obtained. If the sulphate was replaced by 0·9N-sodium chloride, no crystalline product B was precipitated.

Electrolytic Reduction.—This was carried out in a vessel having two mercury pools (anode and cathode), the catholyte volume being approx. 200 ml. The anolyte was 0·1M-sodium carbonate. Anode and cathode compartments were separated by 2% agar gel in saturated potassium chloride solution. The sealed-in current-carrying platinum electrodes were fully covered by the mercury. Both electrode compartments could be flushed with nitrogen. Ground-in fittings in the cathode compartment carried an auxiliary capillary agar bridge close to the mercury surface, to measure the cathode potential, and a syphon, using excess nitrogen pressure to withdraw samples directly into quartz spectrophotometer cells fitted with ground joints.

The catholyte was vigorously stirred at the surface of the mercury pool by a Teflon sealed magnetic stirrer. All preparations were carried out at a current of 1A and cathode area of approx. 30 cm.^{2}. The cathode potential was 0·3 V with reference to the standard calomel electrode before application of the electrolysing voltage, rising to −0·9 V at the beginning of the electrolysis which was continued until the electrode potential rose to −1·1 V. The current efficiency was approx. 30%, as determined by oxidative titration of the resultant solution.

Electrolytic Reduction of Nicotinamide Propiodide.—For 2×10^{-3}M-solutions, the optical density at 265 and 360 mμ increases during electrolysis, the ratio of the two optical densities remaining approximately constant. The product was assumed to be dihydro-derivative B and its concentration was calculated from the molar extinction coefficient. Oxidative titration of the electrolyte solution gave values which were 95% of the calculated. In other experiments the initial concentration was increased to 3×10^{-2}M, and electrolysed with a current of 1 A, with the cathode potential rising from −0·9 to −1·1 V. This cathode potential remained almost constant when the current was varied between 0·5 and 1·5 A. Under these conditions crystals (C) (15%) were formed. The solution was found by titration to contain approx. 85% of the starting material, calculated as the dihydro-derivative. The concentration calculated from the spectrum of the electrolyte solution agreed with this, if the absorption was assumed to be due to the dihydro-derivative B.

The crystals C (Found: C, 65·5; H, 7·7. Calc. for $C_{18}H_{26}O_2N_4$: C, 65·45; H, 7·9%) were easily soluble in ethanol, soluble in hot water and with difficulty in ether or cold water. It recrystallised from carbon dioxide-free hot water as lemon-coloured needles, m. p. 180° (decomp.). The absorption spectrum of a 0·1N-sodium carbonate solution exhibits one peak only, at 355 mμ (ε 10,600, *i.e.*, 5300 per nicotinamide unit of *M* 165). For an ether solution there is a similar spectrum, with the maximum at 345 mμ (owing to the low solubility ε could not be determined). On addition of acid the peak at 355 mμ disappears and a peak at 295 mμ appears. A weighed quantity of the material was dissolved in a small amount of ethanol, diluted with aqueous buffer, and titrated with ferricyanide: the material had 1 reducing equivalent per nicotinamide unit. Calculated on the assumption that the product is a dimer, the titrations gave results between 95 and 102% of the calculated value. The substance is not fluorescent in the solid state or in aqueous carbonate, ethanol, or ether. On acidification of its solution in aqueous carbonate the peak at 355 mμ disappears and a peak at 295 mμ appears.

2×10^{-3}M-Solutions of nicotinamide methiodide and benzylochloride were also reduced electrolytically. Product B was formed.

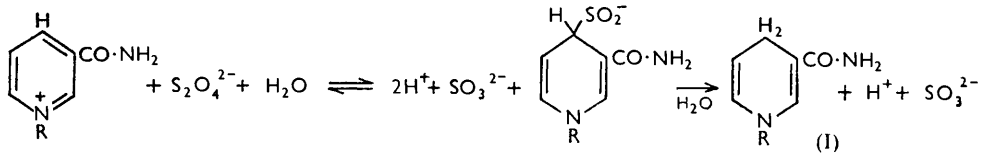
Infrared Spectra.—These were obtained for all three products in KBr discs and for derivative B in Nujol. For the derivatives A and B the spectra are very similar, no main bands exclusive to either occurring, although the relative intensities of several bands differ considerably. The spectrum of the dimer resembles that of the dihydro-derivatives, having in addition a band at 1025 cm.$^{-1}$.

Discussion

The formation of the different products can be understood if we assume that one-electron-equivalent transfers yield free-radical intermediates, the final products differing

from those obtained by processes where two electron-equivalents are simultaneously transferred.

The reduction with dithionite yields the dihydro-derivative A first characterised by Karrer *et al.*[1] Colowick [2] and Swallow [3] have shown that a free-radical intermediate is not formed in this process. The orange intermediate colour is due to a complex formed by the addition of a negative ion, so that the reduction involves a single-step two-electron-equivalent reduction: [2]



Vennesland and Westheimer [4] showed that in the enzymic reduction of DPN^+ a proton is transferred directly from the substrate to the coenzyme, presumably as a hydride ion containing two electron-equivalents. The reduction of DPN^+ by dithionite also affords enzymically active DPNH. Colowick's work makes it likely that product A obtained by dithionite reduction is the *para*-derivative (I).

Reduction by sodium borohydride may yield the same dihydro-derivative A under the conditions described by Brook *et al.*[8] However, as the experimental conditions are modified another dihydro-derivative, B, is obtained. Mathews and Conn [9] found for DPN^+ that reduction with borohydride yields a product which is only partly enzymically active. The evidence in the present paper—its relatively low m. p., similar to that of A, its ultraviolet fluorescent and infrared spectrum, elementary analysis, and oxidation equivalents determined by titration—indicates that product B is another (*ortho*-)dihydro-derivative.

Regarding the mechanism of formation, it is significant that product B is formed in two other processes, whose mechanism involves a free-radical intermediate and involving single-electron-equivalent reduction steps. It was thus first observed when solutions of nicotinamide propiochloride in aqueous solutions of pH 8 containing added ethanol were irradiated with penetrating radiations (*X*- or γ-rays),[5] where it was shown that the reducing agent is the free radical derived from ethanol.

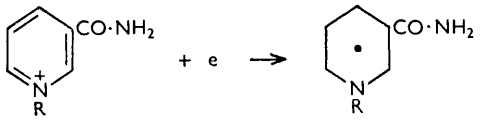
The same dihydro-compound B is obtained in the electrolytic reduction, which proceeds also by single-electron-equivalent transfer steps. Leach *et al.*[10] studied the electrolytic reduction of such model compounds of DPN^+, assuming the products to correspond to that obtained in dithionite (corresponding to enzymic) reduction. However, during electrolysis product B is formed. This agrees with the work of Bacon Ke [11] on the electrolytic reduction of DPN^+ at a mercury cathode. He showed that the product is enzymically inactive, and thus different from the product of dithionite or enzymic reduction.

Product B is thus formed in two different cases where single-electron-equivalent transfer steps, resulting in free-radical intermediates, operate. This suggests that borohydride too is capable under suitable conditions of acting partly by a single-electron transfer.

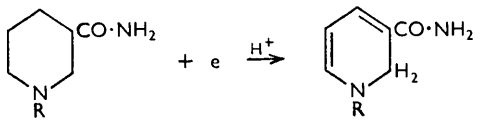
In a process involving free-radical intermediates, the radicals may disproportionate or dimerise. The isolation of the dimer (C) on electrolytic reduction supports the postulated single-electron-transfer mechanism and intermediate free radicals. Its spectrum makes it probable that it is the *para*-derivative. Increasing the concentration of the starting

[9] Mathews and Conn, *J. Amer. Chem. Soc.*, 1953, **75**, 5428.
[10] Leach, Baxendale, and Evans, *Austral. J. Chem.*, 1953, **6**, 395; Leach, *Adv. Enzymol.*, 1954, **15**, 1.
[11] Bacon Ke, *Arch. Biochem. Biophys.*, 1956, **60**, 505; *J. Amer. Chem. Soc.*, 1956, **78**, 3649.

material favours dimer formation in the electrolytic reduction of nicotinamide propiodide. If in the first step at the electrode the radical is formed:



this may be followed at the electrode by:



i.e., formation of product B. If, however, the concentration of starting material is increased, these will be preferentially reduced at the electrode, leaving the radicals free to interact partly by dimerisation.

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Department of Physical Chemistry, Hebrew University,
Jerusalem, Israel. [*Received, September 23rd*, 1957.]