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Radiolysis of Reduced Diphosphopyridine Nucleotide in Aqueous Solution

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The radiolysis of DPNH solutions by 250-kvp X rays has been followed utilizing the absorption at 340 mμ, the corresponding fluorescence at 470 mμ, and the coenzymatic activity with alcohol dehydrogenase. All three parameters are decreased simultaneously and this reduction in DPNH concentration is neither linear nor logarithmic with dose. The coenzyme is protected in the presence of protein; the protection afforded by glutamic dehydrogenase, a DPNH-binding enzyme, is equal to that of serum albumin at the same weight concentration. The radiolysis of DPNH is independent of *p*H over the range 4.8–7.5 and is accompanied by an initial increase in *p*H, 260-mμ absorption, and cyanide reactivity. These observations suggest that DPN is the first product formed which is, in turn, degraded logarithmically. A kinetic model for the radiolysis has been formulated which gives a *G*(-DPNH) of 2.3 in air and 1.2 in oxygen-free nitrogen.

A number of investigations have indicated that ionizing radiation may have a more profound effect on the tissue levels of DPNH[1] than on the oxidized coenzyme. For example, irradiation of grasshopper eggs leads to a decrease in the DPNH-DPN ratio during the period of irradiation, with the ratio returning toward normal following the X-ray exposure (Tahmisian and Wright, 1956). In addition, rat liver concentrations of DPNH are reduced by whole-body doses of X rays that do not change the concentration of DPN appreciably (Eichel and Spirtes, 1955). These findings suggest that irradiation either alters cellular metabolism immediately in favor of a decreased DPNH-DPN ratio or that DPNH is sufficiently sensitive within cells to be attacked directly by the aqueous radicals. As a preliminary to the further elaboration of these *in vivo* observations the comparative radiolysis of reduced and oxidized DPN solutions is described in this report.

Experimental

Solutions of purified DPN and DPNH, obtained from the Sigma Chemical Co. (St. Louis, Mo.), were irradiated with 250-kvp unfiltered X rays at a dose rate of approximately 2000 rad per minute. Ferrous ammonium sulfate dosimeters of the identical geometry were employed and a *G* value of 15 was assumed in the dosage calculations (Swallow, 1960). The solutions were made with deionized, glass-distilled water except in those instances where specific buffer solutions are indicated. The samples and ferrous ammonium sulfate solutions were irradiated in Parafilm-covered Pyrex beakers kept at ice temperature. For the anerobic irradiations, oxygen-free nitrogen was bubbled through the solutions in a sealable manifold prior to X-ray exposure (Laser, 1962).

The optical absorbancy of the solutions was measured in a Beckman ultraviolet spectrophotometer (DU) and the fluorescence in a Bowman-Aminco spectrofluorometer. The coenzymatic activity of DPNH was measured by the reduction in 340 mμ absorbancy following the addition of 30 μg of yeast alcohol dehydrogenase (C. F. Boehringer and Son) at *p*H 7.5 in the presence of 8 mM acetaldehyde. The coenzymatic activity of DPN was measured by the increase in 340 mμ absorbancy following the addition of 30 μg of yeast alcohol dehydrogenase at *p*H 10.1 in the presence of 0.7 M ethanol. The cyanide addition product was determined by adding 1 ml of the original and irradiated solutions to 2 ml of 1.0 M potassium cyanide, and measuring the absorption at 325 mμ.

Purified human serum albumin was a gift from the Protein Foundation and glutamic acid dehydrogenase was obtained from C. F. Boehringer and Son.

Results

The radiolysis of DPNH was followed by its optical absorbancy at 340 mμ, its fluorescence at 470 mμ when excited at 340 mμ, and its coenzymatic capability in the presence of acetaldehyde and alcohol dehydrogenase. From inspection of Figure 1 it is apparent that the radiolysis proceeds to the same extent under each measurement. The degradation of DPNH is neither

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[1] The following abbreviations are used: DPN, diphosphopyridine nucleotide, nicotinamide adenine dinucleotide; DPNH, reduced diphosphopyridine nucleotide, reduced nicotinamide adenine dinucleotide; *G*, molecules converted per 100 ev of energy absorbed.

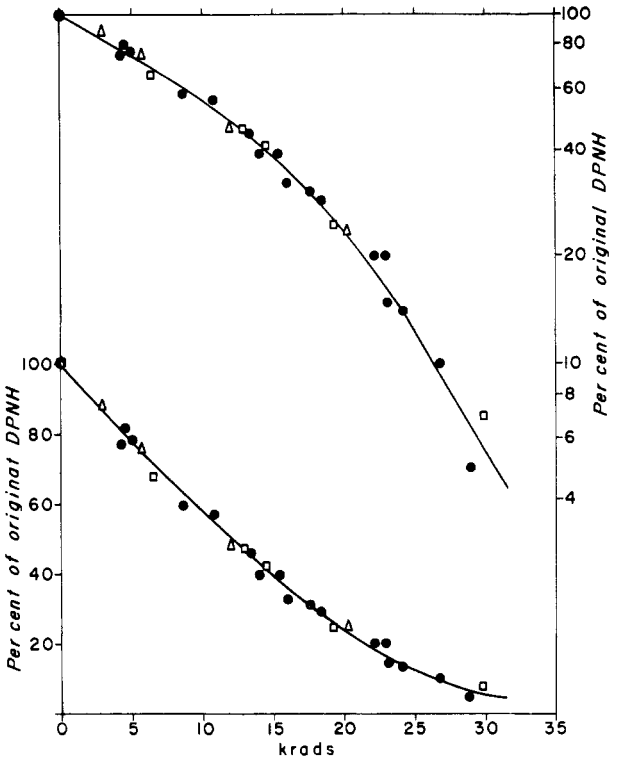

FIG. 1.—Radiolysis of DPNH as a function of dose in kilorads. (●) absorbancy at 340 mμ; (Δ) fluorescence at 470 mμ, 340 mμ excitation; (□) coenzymatic activity with alcohol dehydrogenase and acetaldehyde. DPNH concentration 5.32×10^{-5} M. Upper curve semilogarithmic coordinates, lower curve linear coordinates.

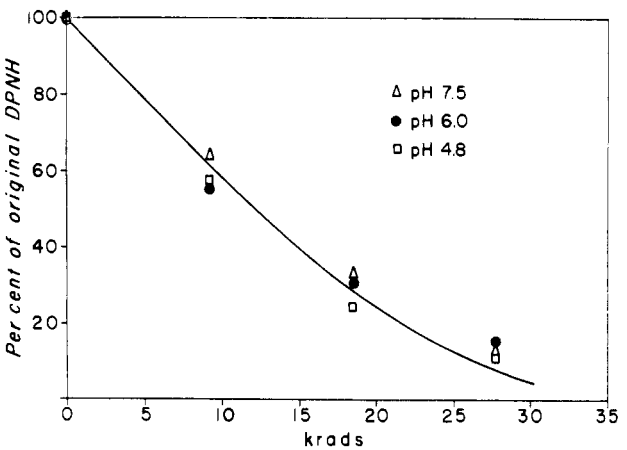

FIG. 2.—Influence of *p*H on the radiolysis of DPNH. Per cent 340-mμ absorbancy vs. dose in kilorads. DPNH concentration 5.32×10^{-5} M. At *p*H 6.0 and 7.5, 10 mM potassium phosphate, and at *p*H 4.8, 10 mM sodium acetate was employed. Same curve drawn as in Figure 1.

logarithmic (upper curve) nor linear with dose (lower curve). The curve drawn in Figure 1 is for a semi-empirical relation discussed below.

The shape of the survival curve is invariant over a concentration range greater and lesser than that shown in Figure 1. Similar curves have been obtained with 2.6×10^{-4} M and 9.7×10^{-6} M DPNH.

The radiolysis is independent of *p*H over the range 4.8–7.5 and is not significantly affected by the addition of 0.01 M potassium phosphate or soduim acetate buffer used in obtaining this data (Fig. 2). The theoretical curve drawn in Figure 2 is taken from Figure 1.

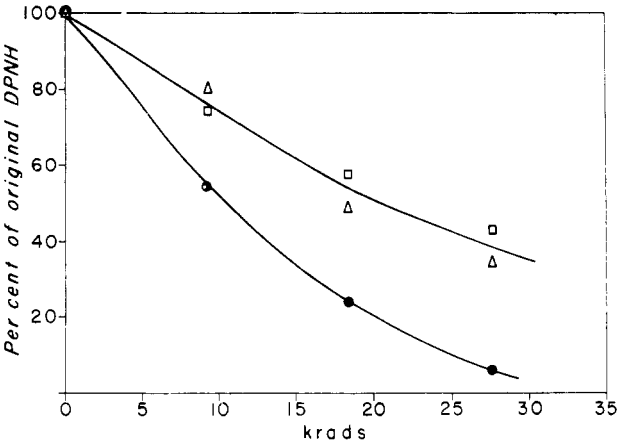

FIG. 3.—Protective effect of proteins on the radiolysis of DPNH. Per cent 340-mμ absorbancy vs. dose in kilorads. (●) 10 mM potassium phosphate, *p*H 7.0; (Δ) serum albumin 3.3 mg/ml in 10 mM potassium phosphate, *p*H 7.0; (□) beef liver glutamate dehydrogenase 3.3 mg/ml in 10 mM potassium phosphate, *p*H 7.0. DPNH concentration 5.32×10^{-5} M.

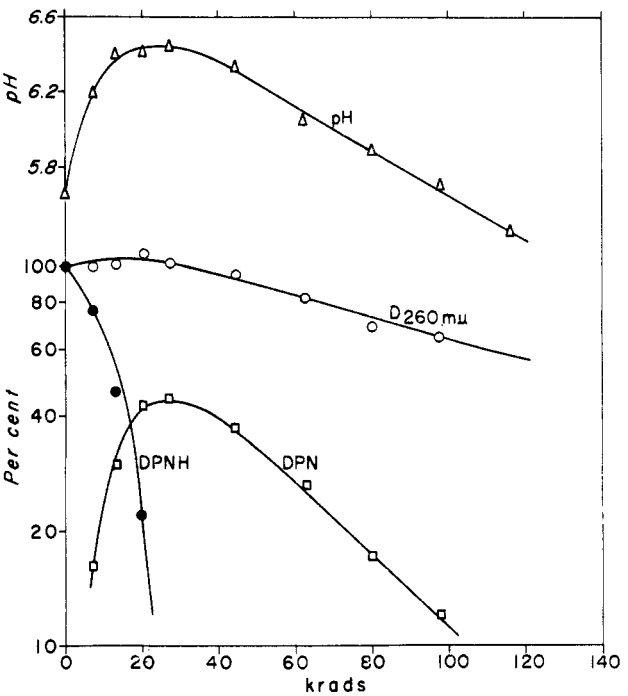

FIG. 4.—Production and logarithmic degradation of DPN following DPNH radiolysis. (●) per cent 340-mμ absorbancy; (□) per cent DPN concentration relative to initial DPNH concentration, determined with alcohol dehydrogenase and ethanol. The change in *p*H (Δ) and 260-mμ absorbancy (○) are also shown as a function of dose in kilorads.

However, the presence of protein will protect the coenzyme from the aqueous radicals. In Figure 3, the radiolysis in 0.01 M phosphate buffer, *p*H 7.0, is compared with that in the presence of 3.3 mg/ml human serum albumin and glutamic acid dehydrogenase. It is noteworthy that glutamic acid dehydrogenase, which is rich in sulfhydryl groups and specifically binds the reduced coenzyme, does not offer significantly greater protection than does serum albumin at the same weight concentration. The amount of DPNH bound to glutamate dehydrogenase at these concentrations of enzyme and coenzyme is greater than 60% of the total as determined by ultracentrifuge separation.

The major, and perhaps sole, product of DPNH radiolysis is the oxidized coenzyme DPN (Fig. 4). The DPN concentration, measured by its reduction in the presence of ethanol and alcohol dehydrogenase, increases to a maximum with the oxidation of DPNH and is in turn degraded. The radiolysis of the formed DPN is logarithmic, which is identical with the radiolysis of pure DPN solutions as shown previously (Myers, 1960). In Figure 4, the optical absorbancy at 260 mμ is seen first to rise as DPNH is oxidized and then to fall as DPN is destroyed. Since the absorbancy of DPN at 260 mμ is greater than that of DPNH, the increase observed initially is consistent with the conversion of DPNH to DPN. In addition, irradiation of DPNH effects a primary rise in *p*H followed by a secondary fall. As irradiation of pure DPN solutions produces a fall in *p*H, this also is consistent with conversion of DPNH to DPN with the latter's subsequent destruction.

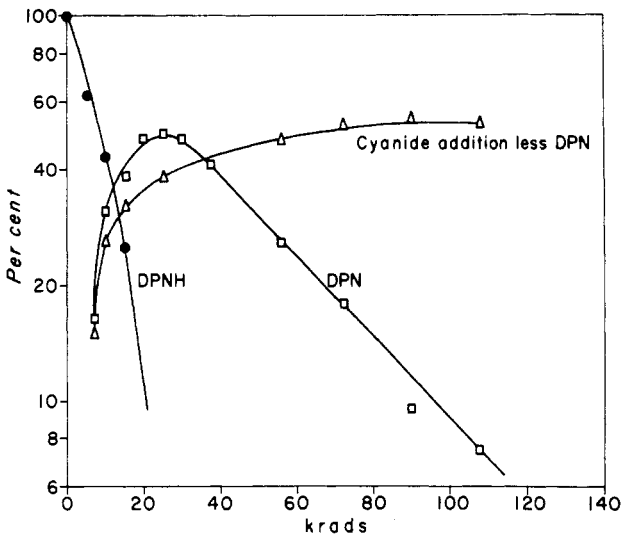

FIG. 5.—The appearance of cyanide reactive molecules following the radiolysis of DPNH. (●) per cent 340-mμ absorbancy; (□) per cent DPN concentration relative to initial DPNH concentration, determined with alcohol dehydrogenase and ethanol; (△) per cent 325-mμ absorption following cyanide addition, relative to that from total theoretical oxidation of initial DPNH less 325-mμ absorption due to DPN.

Figure 5 demonstrates that the degradation of DPNH yields DPN and nicotinamide-ribosides and ribonucleotides capable of reacting with potassium cyanide to form a 325-mμ absorbing addition product (Colowick *et al.*, 1951). The per cent concentration of the nicotinamide ribosides and ribotides was obtained by subtracting that portion of the cyanide-induced absorption due to DPN from the total. During the initial phase of DPNH radiolysis these additional products plus DPN account for nearly all the reduced coenzyme destroyed.

The radiolysis of DPNH is significantly less in an oxygen-free nitrogen atmosphere than it is in air. In Table I the degradation in air and under nitrogen are compared. The shapes of the survival curves in the two atmospheres are similar.

DISCUSSION

The radiolysis of biochemicals in aqueous solution is generally considered to proceed kinetically by one of two mechanisms. Either the products of the radiolytic reaction are assumed not to interact with the aqueous radicals and the reaction is linear with dose, or they are assumed to interact with the same rate constant as the original material and the reaction is logarithmic with dose. For either situation the calculation of ionic yields or "*G*" values is straightforward; in the former instance that dose which accounts for 100% conversion of the original material may be employed, for the latter the 63% dose may be used (Bacq and Alexander, 1961). In those instances where the products of the radiolysis react with the solvent radicals at a different rate than the parent compound, the calculation of yields generally requires an explicit knowledge of the various rate constants or some additional information which will allow an unambiguous estimate to be made. In this communication the radiolysis of DPNH is shown to proceed by a dose dependence that is neither linear nor logarithmic, and indicates such an intermediary mechanism. Product analysis, however, allows the calculation of yield to be made unambiguously.

TABLE I

RADIOLYSIS OF DPNH SOLUTIONS IN AIR AND UNDER OXYGEN-FREE NITROGEN[a]

Dose (krad)	Per Cent of Initial Concentration	
	Air	Nitrogen
4	76	88
8	57	76
12	47	66
16	30	57
20	17	47
32	—	29

[a] Initial DPNH concentration 5.2×10^{-5} M. The degradation of DPNH was followed by the 340-mμ absorbancy.

The general expression for the degradation of substance A going to products B, C, etc., is

$$-dA = \frac{k_1 A\, dX}{k_1 A + k_2 B + k_3 C \ldots} \qquad (1)$$

where k_i is the specific reaction rate constant and X is the radical yield (Allen, 1961). For DPNH, the initial substance, A, is converted stoichiometrically to DPN, B, which in turn is logarithmically degraded to substances C, D, etc. (Figs. 4 and 5) Equation 1 may then be rewritten

$$-dA = \frac{k_1 A\, dX}{k_1 A + k_2 (A_0 - A)} \qquad (2)$$

where A_0 is the initial DPNH concentration. Integration of Equation 2 gives

$$\left(1 - \frac{k_2}{k_1}\right) - \left(1 - \frac{k_2}{k_1}\right)\frac{A}{A_0} - \frac{k_2}{k_1} \ln \frac{A}{A_0} = \frac{X}{A_0} \qquad (3)$$

To calculate the ionic yield set $X = A_0$ and obtain

$$\left(1 - \frac{k_1}{k_2}\right)\frac{A}{A_0} - \ln \frac{A}{A_0} = 1 \qquad (4)$$

The calculation of yield, therefore, requires a knowledge of the ratio k_1/k_2. As an independent means of determining this ratio is presently not available, it must be derived by fitting equation (3) to the data of Figure 1 and finding which value of k_1/k_2 best approximates the experimental points. This was accomplished as follows. An arbitrary value of A/A_0 was chosen (usually 0.2) and the family of curves varying in k_1/k_2 was drawn through the 100% origin and the point corresponding to this arbitrary value. That curve which fitted the data best was then selected and the value of k_1/k_2 was introduced into equation (4). For the data of Figure 1 the best value of k_1/k_2 is equal to 5,

yielding an $A/A_0 = 0.18$ from equation (4). The yields may then be calculated from that dose of X rays corresponding to 82% destruction of the initial DPNH. It is noteworthy that the value of 18% lies midway between 37% for logarithmic survival and 0% for linear survival. Fitting the data of Figure 1 to equation (3) in this manner a G(-DPNH) in air of 2.3 is obtained. When DPNH is irradiated in oxygen-free nitrogen a G(-DPNH) of 1.16 is obtained.

Alternately, the initial near-linear portion of Figure 1 (lower curve) can be extrapolated to zero DPNH concentration and this dosage value used to calculate G(-DPNH). If sufficient data points are available in the initial portion of the curve the G value obtained by extrapolation agrees closely to the value derived by fitting the radiolysis curve to equation (3). The opportunity of using the entire curve rather than its initial linear asymptote is the advantage of the more laborious curve fitting.

The mechanism of the radical reactions with DPNH is unknown but the following sequences are consistent with the yields obtained.

Anaerobic:

$$2\ \mathrm{DPNH} + 2\ \mathrm{OH} \longrightarrow 2\ \mathrm{DPNH^+} + 2\ \mathrm{OH^-}$$

$$2\ \mathrm{DPNH^+} \longrightarrow \mathrm{DPN^+} + \mathrm{DPNH} + \mathrm{H^+}$$

$$\mathrm{DPNH} + 2\ \mathrm{OH} \longrightarrow \mathrm{DPN^+} + \mathrm{OH^-} + \mathrm{H_2O}$$

$$G(\text{-DPNH}) = \frac{G(\mathrm{OH})}{2} = \frac{2.4}{2} = 1.2$$

Aerobic:

$$\mathrm{DPNH} + \mathrm{OH} \longrightarrow \mathrm{DPNH^+} + \mathrm{OH^-}$$

$$\mathrm{H} + \mathrm{O_2} \longrightarrow \mathrm{HO_2}$$

$$\mathrm{DPNH} + \mathrm{HO_2} \longrightarrow \mathrm{DPNH^+} + \mathrm{HO_2^-}$$

$$2\ \mathrm{DPNH^+} \longrightarrow \mathrm{DPN^+} + \mathrm{DPNH} + \mathrm{H^+}$$

$$\mathrm{HO_2^-} + \mathrm{H^+} \longrightarrow \mathrm{H_2O_2}$$

$$\mathrm{DPNH} + \mathrm{H} + \mathrm{OH} + \mathrm{O_2} \longrightarrow \mathrm{DPN^+} + \mathrm{H_2O_2} + \mathrm{OH^-}$$

$$G(\text{-DPNH}) = \frac{G(\mathrm{OH}) + G(\mathrm{H})}{2} = \frac{5.3}{2} = 2.65$$

The yields for H and OH are taken from Allen (1961).

The reaction sequence accounts not only for the yields found but also for the initial increase in pH observed.

An estimate of the 100 ev G value for DPNH was made by Barron (1954) who assumed that the extent of oxidation was directly proportional to the X-ray dose. He obtained a yield of 1.5 in air and also found the yield to be lower in the absence of oxygen. Myers (1960) followed the destruction of DPNH and found that it was nonlinear with increasing dose. Extrapolation of the initial linear portion of his DPNH radiolysis curve to 0% gives an approximate G(-DPNH) of 2.2, in closer agreement with the present value.

The decreased yield observed on the addition of proteins is to be expected since their side chains are known to act as scavengers for aqueous radicals (Swallow, 1960). However, it might be expected that glutamate dehydrogenase which binds DPNH specifically would provide a greater degree of protection than serum albumin. Its failure to do so suggests that the sensitive locus on the dihydronicotinamide ring is equally exposed to radical attack when the coenzyme is bound to enzyme. This finding may be pertinent to *in vivo* irradiations since mitochondrial pyridine nucleotides are presumably bound to specific proteins (Chance and Baltscheffsky, 1958).

It has been suggested that the major product of DPNH radiolysis is DPN (Barron *et al.*, 1954), and this has been quantitatively confirmed in this report. Myers (1960) studied the radiolysis of DPN and found that loss of coenzyme function could be correlated with the destruction of ribose and riboside linkages. He found that the ratio of doses required to reduce the nicotinamide-ribose bond and coenzyme activity to 37% of their initial values was 2.6. Analysis of our cyanide addition data (Figure 5) sets the value of this ratio to 5 and indicates a more radioresistant nicotinamide-ribose linkage.

Since DPNH is more radiosensitive than DPN and since the first product of DPNH radiolysis is DPN, the decrease in DPN-DPNH ratio observed during the irradiation of grasshopper eggs (Tahmisian and Wright, 1956) is consistent with a direct radical attack on the coenzyme. However, the ability of the cellular contents to protect molecules from the destructive action of ionizing radiation is well recognized (Adelstein, 1962) and an alternate hypothesis based on changes in oxidative metabolism is equally valid. The choice of alternatives will require further studies with cell fractions and homogenates.

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