Pyridine nucleotide oxidation and reduction in biological systems is frequently catalyzed by flavin enzymes. A 2-eq reduction of the pyridine ring and the addition of a proton results in the formation of a higher energy compound (1). The mechanism of this process has been the subject of earlier investigations in this laboratory. Photooxidation of the nicotinamide adenine dinucleotide NADP+ was shown to occur in the presence of either of two synthetic pigments structurally unrelated to chlorophyll (2) and the flavoprotein ferredoxin-NADP reductase (2). The formation of a biologically active reduced product was found to be dependent on the presence of the enzyme. This finding suggested to us the possibility that radiolysis could be used to produce a similar reduction of NADP+ to the biologically active product NADPH.

The irradiation of neutral water by x-rays, γ-rays, or high energy electrons generates the following species within 10−8 s from the time of energy absorption (3):

\[ \text{H}_2\text{O} \rightarrow e_{\text{aq}}, \text{OH}, \text{H}, \text{H}_2\text{O}, \text{OH}_{\text{aq}}, \text{H}_2\text{O}_{\text{aq}} \]

Among the products are strong reducing and oxidizing agents. The strongest reducing agents are the hydrated electron, \( e_{\text{aq}} \) (\( E^0 = -2.7 \) volts), and the hydrogen atom (\( E^0 [\text{H}/\text{H}^+] = -2.1 \) volts); powerful oxidizing agents include the \( \text{OH} \) radical (\( E^0 [\text{OH}]/[\text{OH}^-] = +2.8 \) volts) (4).

Oxidation-reduction properties of pyridine-containing compounds have been investigated extensively. Kosower (5) reviewed the studies on pyridine derivatives including the pyridine nucleotides. Radiolysis in the absence of enzymes produces a single equivalent reduction of the pyridine ring via a radical mechanism (6, 7); two of the radicals formed then react to yield a stable species thought to be a reduced dimer. The final product is inactive with the lactate and ethanol substrate dehydrogenases (6) and with spinach ferredoxin-NADP reductase flavoprotein (this paper). This reduction pathway has been proposed to describe nonenzymatic reductions of pyridine compounds (5, 8, 9).

The present paper reports the production of NADPH during steady state irradiation of NADP+ containing solutions in the presence of a flavoprotein with ethanol or methanol to act as scavengers for hydroxyl radicals (10). The presence of the flavoprotein, ferredoxin-NADP reductase, is required for the formation of NADPH; in the absence of enzyme, the biologically inactive dimer (5–7) is produced.

**METHOD**

Radiolysis is performed in a phosphate-buffered aqueous solution containing 0.1 to 1 mm NADP+ and 0.1 to 1 mm ethanol or methanol. The flavoprotein is added as needed in 50 mM phosphate buffer, pH 7.4. Solutions are irradiated in rectangular quartz cells (30 × 10 × 5 mm). Oxygen is removed by evacuation or by bubbling argon through the solutions and subsequent measurements are made under anaerobic conditions. The irradiation employs either a Philips Electronics x-ray tube operating at 75 kv or a Van de Graaff accelerator operating at 2 m.e.v. The dose rates are, respectively, 170 or 6000 rads per s; the dosage is measured with an air-saturated solution of 10 mm FeSO₄ in 0.8 M H₂SO₄ (11, 12).

Optical spectra are measured in a Cary model 14 spectrophotometer before and after irradiation; the fluorescence spectra in a Hitachi Perkin-Elmer MPF-2A spectrophotometer. NADP+ and NADPH are used as purchased from Sigma Chemical Co. Ferredoxin-NADP reductase is purified from spinach according to Forti and Sturani (13) and stored at 20°C.

**RESULTS**

Dose Rate and Enzyme Concentration—The irradiation of an aqueous solution of NADP+ in the presence of ethanol or methanol plus the flavoprotein ferredoxin-NADP reductase yields two reduced products whose relative amounts depend upon the enzyme concentration and the dose rate. In the absence of the enzyme only the dimer, referred to as (NADP)₂, appears. It is presumably formed from two pyridine radicals as suggested by Land and Swallow (7). With the reductase enzyme present at 50 to 100 μM, x-ray irradiation at 170 rads per s produces only...
Table I

| Enzymatic versus nonenzymatic products of pyridine nucleotide x-irradiation | Products<sup>b</sup> |
|---|---|
| <sup>a</sup>Irradiation in 2 ml of 0.25 mM phosphate buffer, pH 7.4, containing 100 μM NADP<sup>+</sup>, 0.5 mM ethanol. Dose ≈17 krads in 100 s-170 rads per s. | NADPH | (NADP)<sub>2</sub> |
| 0 | nil | 38 ± 2<sup>6</sup> |
| 100 | 38 ± 2 | <2<sup>6</sup> |

<sup>a</sup>Irradiation in 2 ml of 0.25 mM phosphate buffer, pH 7.4, containing 100 μM NADP<sup>+</sup>, 0.5 mM ethanol. Dose ≈17 krads in 100 s-170 rads per s.

<sup>b</sup>Ferricyanide titration based on 2 reducing eq per mole.

**Fig. 1.** Radiolysis yield of NADPH as a function of NADP<sup>+</sup> concentration. Conditions as for Table I; reductase 80 nM; x-ray dose 17 krads in 100 s. A similar curve describes (NADP)<sub>2</sub> yield in the absence of the enzyme ferredoxin-NADP reductase.

**Fig. 2.** Absorbance spectra of NADP<sup>+</sup> and irradiation products (NADP)<sub>2</sub> and NADPH. Reaction conditions as for Table I; light path 1 cm.

**Fig. 3.** Ferrodoxin-NADP reductase-coupled ferricyanide oxidation of NADPH versus (NADP)<sub>2</sub>. t = lifetime of reduced nucleotide in presence of ferrodoxin-NADP reductase with 60 μM Fe(CN)<sub>6</sub><sup>4-</sup> as oxidant.

The enzymatically active product, NADPH. The production of NADPH and (NADP)<sub>2</sub> with x-rays is shown in Table I and in Fig. 1. Van de Graaff irradiation at 6000 rads per s with the reductase concentration at 500 nM yields roughly equal amounts of NADPH and (NADP)<sub>2</sub>. The higher dose rate presumably results in the requirement for greater enzyme concentration to prevent the accumulation of free radicals and their dimerization to (NADP)<sub>2</sub>. The (NADP)<sub>2</sub> is not converted to NADPH by treatment with the reductase after irradiation.

The products of radiolysis have been characterized by the following five properties.

1. Optical spectra of the enzymatic and nonenzymatic products are shown in Fig. 2. The spectrum of the former coincides with that of NADPH. The nonenzymatic product (NADP)<sub>2</sub> has a broader absorption band in the 340-nm region and an extinction coefficient approximately 15% less than the value for NADPH, i.e. \( \varepsilon_{240}^{NADP} = 6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \) (14).

2. Fluorescence excitation of the radiolysis products in the 340-nm absorption region gives different results in the presence and absence of enzyme. The enzymatic product has the 460 nm emission characteristic of NADPH in both intensity and position whereas the nonenzymatic product presumed to be (NADP)<sub>2</sub> has an emission maximum at 445 nm with only one-tenth the intensity of the enzymatic product.

3. Stability in solution is less for (NADP)<sub>2</sub> than for NADPH. Their respective lifetimes in the irradiated solutions are about 2 and 18 hours. Neither is particularly sensitive to O<sub>2</sub> and both are acid-labile. On the addition of HCl or by bubbling CO<sub>2</sub> through the solution, both undergo rapid decomposition. The acid sensitivity caused us to select conditions which would hold the pH after irradiation in the region between 6.8 and 7.6. In the dilute buffer 0.25 mM phosphate, pH 7.6, a drop of about 0.5 pH unit occurred during the irradiations. As shown in Fig. 1, this change was not important in the determination as indicated by the identical data for NADPH accumulation in 10 mM phosphate buffer, pH 7.6, in which the pH remained constant.

4. Enzymatic oxidation of reduced products from radiolysis by ferricyanide followed by decrease in absorption at 340 nm is shown in Fig. 3. Fig. 4 illustrates the effect of the reductase concentration during irradiation on the fraction of the NADP<sup>+</sup> converted to the enzymatically active NADPH. (NADP)<sub>2</sub> dimer can be oxidized by ferricyanide but the rate is enzymeb-independent. With equal equivalents of (NADP)<sub>2</sub> and ferricyanide the oxidation is completed in about 15 min. The prod-
indicating this product to be \( \text{XAD}^+ \text{H} \).

When one accounts for the difference in extinction coefficients in these experiments, the data are within the experimental error. Therefore the two solutions were assayed for residual \( \text{NADP}^+ \) by the addition of an excess of glucose 6-phosphate and its dehydrogenase and the total free oxidized pyridine nucleotide measured by the increase in absorption at 340 nm. The concentration in the experiment containing the enzyme during irradiation showed a residual \( \text{NADP}^+ \) of 56 \( \mu \text{M} \), whereas the enzyme-free sample contained only 33 \( \mu \text{M} \). These data support the contention that the irradiation product in the absence of enzyme is in fact a dimer. Although the recombinations are not quantitative, 103\% in the presence of the dimer and 91\% in the presence of the enzyme, when one accounts for the difference in extinction coefficients in these experiments, the data are within the experimental error.

Enzyme and Nucleotide Specificity—The flavoprotein ferredoxin-NADP reductase is necessary for the formation of the biologically active reductant, \( \text{NADP}^+ \), as was shown by substituting the heat-denatured for the native enzyme and by attempting to substitute free \( \text{FAD} \) for the enzyme. Flavoprotein which had been allowed to stand at room temperature for 48 hours in the presence of air lost both the ability to catalyze the oxidation of \( \text{NADPH} \) in the presence of ferriyanide and the ability to yield the enzymatically active product during irradiation. Free \( \text{FAD} \) at concentrations of 50 \( \mu \text{M} \) to 100 \( \mu \text{M} \) was similarly ineffective.

The specificity for \( \text{NADP}^+ \) versus \( \text{NADPH}^+ \) of the flavoprotein during irradiation was examined using the irradiation conditions reported in Table I and Fig. 4. The 30 \( n \text{M} \) concentration of enzyme found effective for \( \text{NADPH} \) did not result in the formation of \( \text{NAD}^+ \). When the enzyme concentration was raised to 1 \( \mu \text{M} \), as opposed to 30 \( n \text{M} \), an appreciable yield of \( \text{NADH} \) was observed. Thus the selectivity of the flavoprotein for the specific nucleotide is the same in irradiation reduction as in reactions with the more conventional reducing agents (16).

**Fig. 4.** Effect of reductase concentration on fraction of nucleotide reduced to \( \text{NADPH} \). Irradiation as for Table I. Products assayed by \( \Delta 340 \text{ nm} \) in presence of 100 \( \mu \text{M} \) ferriyanide and ferredoxin-NADP reductase. \% \( \text{NADPH} = A(NADPH)/[A(NADPH) + A((\text{NADP})_2)] \), where \( A \) is absorbance.

The biological reduction of \( \text{NADP}^+ \) to \( \text{NADPH} \) by the flavoprotein ferredoxin-NADP reductase requires two electrons and a proton. The flavin prosthetic group of the enzyme has three documented oxidation states: the oxidized, the one-electron reduced semiquinone, and the two electron, or fully reduced (5, 15, 16). The flavoprotein can extract electrons from one-electron donors and transfer them to two-electron acceptors, such as \( \text{NADP}^+ \). The way in which the electrons are transferred, in particular the roles of one- and two-electron reduced states of the enzyme in biological oxidoreduction, is still unclear.

Our experiments show that the reduction of \( \text{NADP}^+ \) to the biologically active \( \text{NADPH} \) can be initiated by ionizing radiation coupled with the flavin enzyme ferredoxin-NADP reductase. In the absence of the enzyme a biologically inactive reduced compound, probably the dimer \( (\text{NADP})_2 \), is formed. Land and Swallow have given rate constants for the production of \( (\text{NAD})_2 \) via hydrated electrons (7):

\[
\begin{align*}
\text{NAD}^+ + e_{\text{aq}} & \rightarrow \text{NAD} \quad k = 2.5 \times 10^{10} \text{ M}^{-1} \text{s}^{-1} \\
2\text{NAD}^- & \rightarrow (\text{NAD})_2 \quad k = 5.6 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}
\end{align*}
\]

Almost identical results were found when \( \text{NAD}^+ \) was replaced by \( \text{NADP}^+ \).

In our experiments, the number of hydrated electrons reacting with enzyme molecules is less than the number reacting with \( \text{NADP}^+ \) molecules because of the high ratio of \( \text{NADP}^+ \) to ferredoxin-\( \text{NADP} \) reductase and the large rate constant of the \( \text{NADP}^+ \)-producing reaction. Direct reduction of the enzyme by either hydrated electrons or alcohol is unlikely under our working conditions in view of the rate constants demanded at the reactant concentration used. The direct reduction rate by hydrated electrons would have to be \( k = 10^{14} \text{ M}^{-1} \text{s}^{-1} \) with several orders of magnitude beyond diffusion limits (17). Direct reduction by alcohol radicals is similarly ruled out in view of their high reaction rate, \( k \approx 10^9 \text{ M}^{-1} \text{s}^{-1} \) with \( \text{NADP}^+ \).

The most direct interpretation of the enzymatic interception of dimerization is a disproportionation of the pyridine radicals. The nature of the enzyme-substrate complexes formed during this reaction cannot be clarified at present; more refined techniques are needed. The possibility of binding singly reduced \( \text{NADP}^- \) to enzyme, however, or more generally the occurrence of a singly reduced bound state, immediately presents itself.

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REFERENCES

1. Vennesland, B., and Westheimer, F. H. (1954) in The Mechanism of Enzyme Action (W. D. McElroy and B. Glass, eds) pp. 327-388, The Johns Hopkins Press, Baltimore

2. Greenbaum, E., Austin, R. H., Frauenfelder, H., and Gunsalus, I. C. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1273-1276

3. Matheson, M. S., and Dorfman, L. M. (1969) Pulse Radiolysis, pp. 63-64, M.I.T. Press, Cambridge

4. Baxendale, J. H. (1964) Rad. Res. Suppl. 4, 114-138

5. Kosower, E. M. (1962) Molecular Biochemistry, pp. 150-227, McGraw-Hill, New York

6. Swallow, A. J. (1955) Biochem. J. 61, 197-203

7. Land, E. J., and Swallow, A. J. (1968) Biochim. Biophys. Acta 162, 327-337

8. Leach, S. J., Baxendale, J. H., and Evans, M. G. (1953) Aust. J. Chem. 6, 395-408

9. Underwood, A. L., and Burnett, R. W. (1973) in Electroanalytical Chemistry (A. J. Bard, ed), Vol. 6, pp. 27-55, Marcel Dekker, New York

10. Dorfman, L. M., and Taub, I. A. (1963) J. Am. Chem. Soc. 85, 2370-2375

11. Allen, A. O., and Rothschild, W. G. (1957) Radiat. Res. 7, 591-602

12. Hart, E. J., and Anbar, M. (1970) The Hydrated Electron, pp. 202-203, John Wiley and Sons, New York

13. Forti, G., and Sturani, E. (1968) Eur. J. Biochem. 3, 461-472

14. Horlicker, B. L., and Kornberg, A. (1948) J. Biol. Chem. 175, 385

15. Mahler, H. R., and Gunders, F. H. (1971) Biological Chemistry, 2nd Ed, pp. 441-448, Harper and Row, New York

16. Shin, M. (1968) in Flavins and Flavoproteins (K. Yagi, ed) pp. 114, University of Tokyo Press, Tokyo

17. Eigen, M., and Hammes, G. (1965) in Advances in Enzymology (F. F. Nord, ed) pp. 9-13, Interscience Publishers, New York
Enzymatic reduction of nicotinamide adenine dinucleotide phosphate induced by radiolysis.

S S Chan, T M Nordlund, H Frauenfelder, J E Harrison and I C Gunsalus

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