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THE PHOTOCHEMICAL OXIDATION OF DPNH WITH RIBOFLAVIN PHOSPHATE

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Recently this laboratory has reported studies on the non-enzymatic catalysis of oxidation by flavin coenzymes of various nitrogen compounds in light.[1,2] In an extension of these studies to other coenzymes, we have found that reduced diphosphopyridine nucleotide (DPNH) can also be efficiently oxidized photochemically by

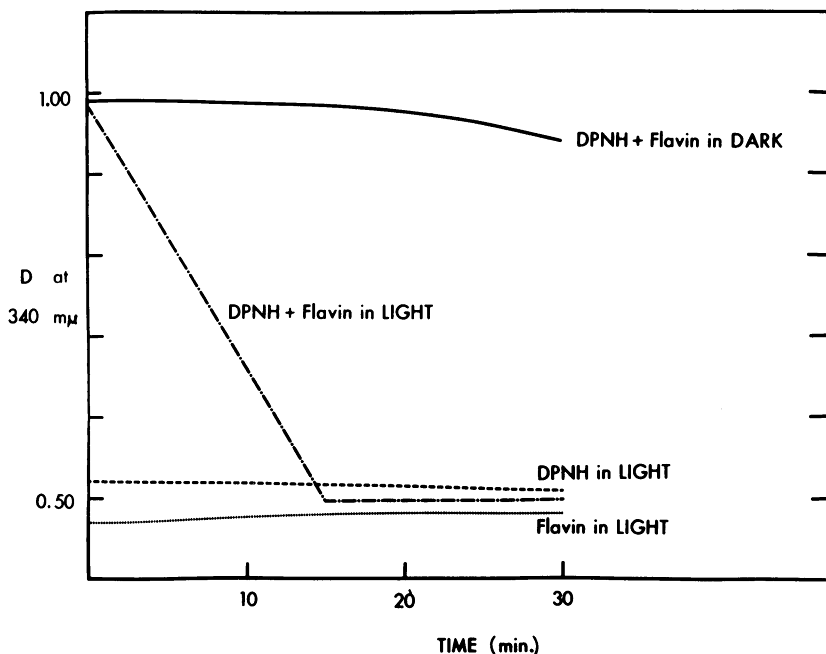


FIG. 1.—Oxidation of DPNH by riboflavin phosphate in light under aerobic conditions. Quartz cuvettes contained 0.27 μmole of DPNH (Sigma Chemical Co.) and/or 0.25 μmole of riboflavin phosphate (Sigma Chemical Co.) in 3.0 ml of 0.075 *M* potassium phosphate buffer (0.0001 *M* in Mg^{++}), pH 7.8. For illumination, cuvettes were placed 2 cm from 36-in. General Electric "black light" fluorescent lamp, and "dark" controls covered with black paper. Optical densities at 340 mμ measured with Beckman DU spectrophotometer.

riboflavin phosphate in the absence of apoenzymes or any other organic compounds. Typical results are presented in Figure 1. Thus, it will be seen that when a solution

of DPNH plus riboflavin phosphate is illuminated aerobically, the DPNH rapidly disappears as evidenced by the loss of the characteristic 340 mμ absorption. On the other hand, the level of DPNH is not significantly decreased when the same mixture is kept dark or when the DPNH is illuminated in the absence of the flavin. The 340 mμ absorption of flavin itself remains constant in these experiments.

Following exposure of the DPNH-riboflavin phosphate mixture to light and the disappearance of the 340 mμ absorption, treatment of the solution with dithionite[3] regenerates the DPNH quantitatively, as shown in Table 1. Further illumination

TABLE 1

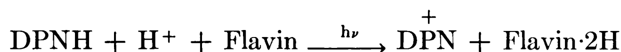
PHOTOOXIDATION OF DPNH WITH RIBOFLAVIN PHOSPHATE AND QUANTITATIVE REGENERATION OF THE DPNH BY DITHIONITE TREATMENT

	D 340 mμ
DPNH + Flavin:	
(a) Before illumination	0.383
(b) After illumination	0.007
(c) Solution (b) after dithionite treatment	0.385
(d) Solution (c) after second illumination	0.007

Rectangular quartz cuvettes contained 0.27 μmole DPNH and/or 0.25 μmole riboflavin phosphate in a total volume of 3.0 ml of 0.075 *M* potassium phosphate buffer (0.0001 *M* in Mg^{++}), pH 7.8. Reagent "blank" for spectrophotometric measurements was the solution containing only the flavin. 0.7 ml. of 0.2 per cent dithionite (in 1% $NaHCO_3$) employed for DPNH recovery. Conditions for illumination same as given in Fig. 1.

of this same solution leads again to oxidation of the DPNH. It can be concluded, therefore, that the loss of two electrons is the major, if not the only, chemical change incurred by the DPNH molecule when illuminated in the presence of the flavin.

It now became pertinent to determine whether the riboflavin phosphate is reduced to its leuco form concurrently with the photoxidation of the DPNH. Under the aerobic conditions of the experiments described in Figure 1, it is not possible to isolate the reduced form of the flavin because of its rapid oxidation by molecular oxygen.[2] Thus, as seen in Figure 2, the oxidized form of the riboflavin phosphate is recovered almost quantitatively from the reaction mixture when the oxidation of the DPNH is complete. Consequently, the photooxidation of DPNH with the flavin was carried out in the absence of oxygen by flushing the system with nitrogen prior to illumination. Upon exposure to light this anaerobic DPNH-flavin mixture immediately blanched, as evidenced by the loss of both the yellow color and the fluorescence. Spectroscopic analysis also showed that the 445 mμ peak of the flavin had disappeared. When oxygen was again admitted to the system, the yellow color and fluorescence characteristic of the oxidized flavin reappeared immediately. No change in the fluorescence and color of the control solution containing only the flavin could be observed during this experiment. These results demonstrated unequivocally that the riboflavin phosphate is the electron acceptor in the photooxidation of the DPNH and also that the DPNH is the predominant, and perhaps only, source of electrons in the system. The stoichiometry of the electron transfer can therefore be described by the following equation:



$$\text{DPNH} + \text{H}^+ + \text{Flavin} \xrightarrow{h\nu} \overset{+}{\text{DPN}} + \text{Flavin}\cdot 2\text{H}$$

From our previous findings[2] it may be presumed that the subsequent reoxidation of the leuco form of the flavin with molecular oxygen yields hydrogen peroxide.

Our earlier studies showed that the riboflavin phosphate can serve a dual function

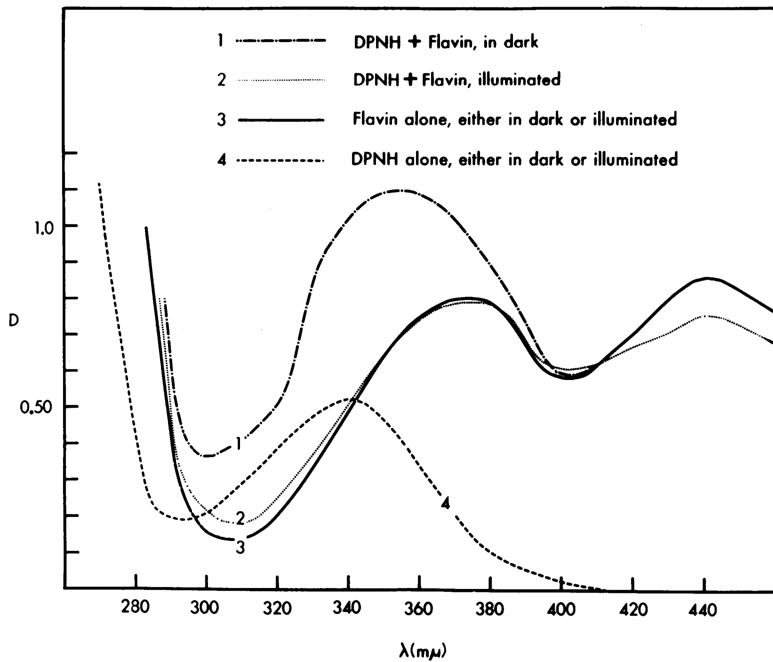


FIG. 2.—Absorption spectra of DPNH, riboflavin phosphate, and DPNH plus riboflavin phosphate before and after illumination under aerobic conditions. Absorption spectra of solutions employed in experiment of Figure 1 were measured in Beckman DU spectrophotometer.

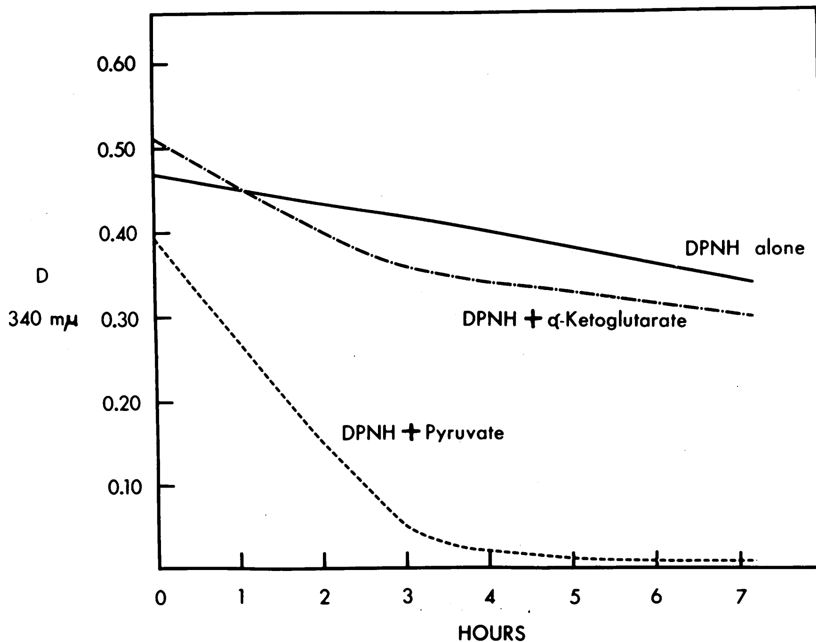


FIG. 3.—Photooxidation of DPNH in presence of pyruvate or α-ketoglutarate. 0.27 μmole of DPNH and 30 μmoles of sodium pyruvate or sodium α-ketoglutarate (Nutritional Biochem. Corp.) dissolved in 3.6 ml of 0.075 M potassium phosphate (0.0001 M in Mg^{++}), pH 7.8, and illuminated as described in Figure 1.

in photooxidations, acting both as the electron acceptor and, in its photoactivated state, as the source of the activation energy for the dehydrogenation of aliphatic nitrogenous "substrates." There was no indication that the latter compounds were photoactivated during this process. DPNH, on the other hand, can be photoactivated, as evidenced by its ability to fluoresce, and it is not unlikely that *both* the DPNH and the riboflavin phosphate are therefore activated in the electron transfer reaction described above. Indeed, the most direct evidence that DPNH itself becomes reactive in light is provided in other experiments, summarized in Figure 3, in which we have found that "black light" illumination of a buffered mixture of DPNH and pyruvate leads to rapid oxidation of the coenzyme. Furthermore, it should be noted that this photoactivated removal of H from the DPNH in the presence of the keto acid exhibits considerable structural specificity, as demonstrated by the fact that the rate of reaction with α-ketoglutarate is appreciably lower than with pyruvate.

The photochemical oxidation of DPNH by riboflavin phosphate reported here recalls the observations by Singer and Kearney[4] on the non-enzymatic reduction of ferricytochrome C by DPNH, mediated by various flavins, and the more recent studies of Suelter and Metzler[5] on the non-enzymatic oxidation of DPNH by riboflavin derivatives. Although no mention of any effect of light was made in either report, it is not unlikely that these reactions could also be stimulated by light. The photochemical oxidation of DPNH by flavin demonstrated by our experiments also suggests a possible mechanism for the phenomenon of "photo-reactivation" described by Dulbecco.[6] He has found that when bacteria are damaged by ultraviolet light, they can be reactivated by light of longer wavelengths and that the action spectrum of this reactivation has a peak at about 3,500 Å. The fluorescent light source employed in our studies has a maximum emission of this same wavelength.

Of the non-chemical forms of energy, light can be most efficiently transformed into chemical energy without profoundly altering the receptor system or its surroundings. Consequently, the capture of light energy and its translation into chemical work may have been an event of considerable importance in the evolution of organic and biochemical reactions. Such absorbed light could have provided the energy of activation required for the catabolism of primordial organic compounds as well as the energy for driving anabolic reactions. In the former case both the compounds produced and the energy liberated could have been used, under favorable conditions, for the synthesis of larger and more complex molecules. If light played such a role, it is probable that compounds which possessed the ability to absorb light and function as catalysts in primitive organized systems may have continued to function catalytically in biochemical reactions even after the origin of proteins.

Both riboflavin and DPNH, ubiquitous in their occurrence, are prime examples of such catalytically active molecules and the significance of their photosensitivity must be considered in any comprehensive hypotheses of chemical evolution. As recently reported from this laboratory,[2] a number of simple nitrogenous compounds are formaldehydogenic when exposed to light and riboflavin phosphate. Relevant to this finding, it was pointed out that not only have formaldehyde and its products been underscored in theories of the origin of life,[7] but that the same compounds which were found to be formaldehydogenic have also been proposed as primordial

substances.[8] Our current demonstration of the photoreactivity of DPNH, still another vitamin derivative, lends an even wider perspective to the plausible implications of such compounds in evolutionary processes. Being the most abundant and universal of the contemporary respiratory coenzymes, the pyridine nucleotides also lie closest to the metabolic "substrate levels" and are both the initial electron acceptors in catabolic processes as well as the final hydrogen donors in synthetic reactions. The simple experiments described here provide a model system for demonstrating how the interplay of light and a reduced coenzyme can lead to the transfer of electrons either in the "substrate direction" or toward other oxidants, including molecular oxygen. In this respect, the action of light could modify a delicate balance between processes of synthesis and degradation both in primordial and contemporary life. Further experiments on the photodynamic reactions of vitamins, nucleotides, and other heterocyclic nitrogenous bases are now in progress.

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ELEMENTS OF A GENERAL THEORY OF ENZYMATIC HYDROLYSIS

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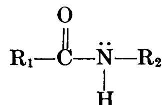
The following propositions seem to us to form a suitable basis for a general or, at least, a fairly general theory of the mechanism of enzymatic hydrolysis:

First proposition. In practically all (in fact, in all but one) fundamental types of biochemical substrates undergoing enzymatic hydrolysis the bond which is hydrolyzed carries formal positive charges on its two extremities. We shall call it a "dipositive bond."

This notion of a dipositive bond may need an explanation. Let us consider, as an example, the peptide bond:



This bond is formed of a basic skeleton of single localized (σ) links, upon which there is superposed a *system of mobile or π electrons.* This system is composed in this case of four electrons: two electrons of the double bond of the C=O link and two electrons of the lone pair of the nitrogen atom. These four electrons constitute a resonating system and, as a general rule for cases where there is a lone pair containing