Photochemistry of N^1-Methylnicotinamide Salts*

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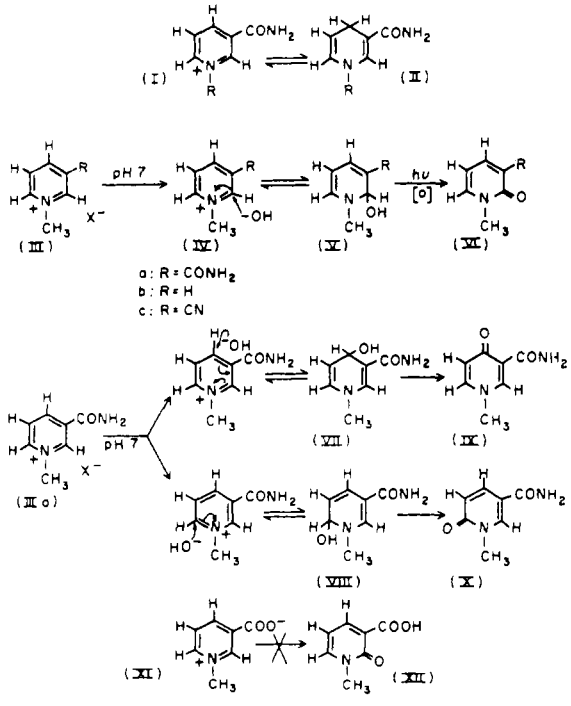
ABSTRACT: The formation of an oxidation product, 1,2-dihydro-1-methyl-2-oxonicotinamide, as a result of ultraviolet irradiation of N^1-methylnicotinamide salts at pH 7, causes an apparent increase in absorbancy at the 330-nm region and suggests that the 2-hydroxy-1,2-dihydro derivative is an intermediate. Spectroscopic evidence indicates that this reaction course is favored at neutral pH and in the presence of ethanol; acetaldehyde and oxygen have no apparent effect. Neither trigonelline nor pyridinium salts form similar photooxidation products, suggesting that a dihydro derivative is one, but not the only, requirement for this reaction. Since both nicotinonitrile methyl iodide and nicotinamide salts form similar photoproducts, a cyano group and an amide group probably exert similar influence on the reaction.

In our study of the photochemistry of biologically important compounds, an investigation of the mechanism of the photochemical reaction of NAD[1] and NADP has been initiated. When these compounds are exposed to ultraviolet light they become inactivated (Runnstrom *et al.*, 1934; Wells, 1956). When linked with certain dehydrogenases, NAD and NADP catalyze biological redox reactions which involve the reduction and re-formation of the nicotinamide moiety (I, Scheme I; Karrer and Warburg, 1936). 1,4-Reduction of NAD or NADP (II; Pullman *et al.*, 1954; Loewus *et al.*, 1955) elicits a characteristic change of ultraviolet absorbancy; *i.e.*, an increase of the absorbancy at 340 nm (λ_{max} for II) together with a partial decrease at 260 nm (λ_{max} for the adenine moiety and I). Since irradiation of NAD with γ- (Swallow, 1953) or X-rays (Swallow, 1955; Barron *et al.*, 1954) in deoxygenated aqueous solutions containing ethanol resulted in increased absorbancy at 340 nm, it was concluded that reduced NAD was formed. However, this irradiation product no longer exhibited enzymatic activity. Similarly, preliminary findings in the ultraviolet irradiation of NAD and NADP (Carter, 1950; Seraydarian *et al.*, 1954; Seraydarian, 1955, 1956; Ekert and Monier, 1958) indicated that photochemical changes take place only in the nicotinamide moiety, with adenosine diphosphate, adenylic acid, adenine, and nicotinamide as photoproducts. Since the photochemical changes were confined to the nicotinamide moiety, N^1-methylnicotinamide salts (IIIa) were chosen as model compounds in these studies (Kaplan, 1960). We have observed an increase in absorbancy at 340 nm, maximum at 325 nm, during ultraviolet irradiation of IIIa; however, the photoproduct responsible for it is 1,2-dihydro-1-methyl-2-oxonicotinamide (VIa), an oxidation rather than a reduction product.

SCHEME I



Materials and Methods

Materials

Table I lists the compounds which were prepared according to the references given.

N^1-Methylnicotinonitrile Iodide (IIIc). Nicotinonitrile (1 g) was dissolved in 10 ml of ethanol, and 2 ml of methyl iodide was added. The solution was allowed to stand at room temperature overnight. The product crys-

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[1] See *Biochemistry 5*, 1445 (1966), for nomenclature.

TABLE I

Compounds	Absorbancy Max (nm) $\epsilon \times 10^{-3}$		mp (°C)
N^1-Methylnicotinamide iodide (IIIa, Karrer *et al.*, 1936)	265	4.48[a]	205–206
N^1-Methylnicotinamide chloride (IIIa, Karrer *et al.*, 1936)	265	4.48[b]	238–239
Trigonelline (XI, Turnau, 1905)	265	4.07[b]	218 dec
N-Methylpyridinium Iodide (IIIb, Kosower and Skorcz, 1960)	256	4.23	116–117
1,2-Dihydro-1-methyl-2-oxonicotinamide (VIa, Pullman and Colowick, 1954)	325	9.11	223–224
1,6-Dihydro-1-methyl-6-oxonicotinamide (X, Pullman and Colowick, 1954)	258 293	14.6 5.14	214–215
1,4-Dihydro-1-methyl-4-oxonicotinamide (IX, Wieland *et al.*, 1961)	256 285	14.8 5.89	181–182

[a] Hochberg *et al.* (1945). [b] Moores *et al.* (1951).

OD 265 nm		OD 325 nm		pH			
Initial	Final	Initial	Final	Initial	Final	Buffer (ml)	Ethanol (ml)
0.49	0.36	0.01	0.01	5.5	3.3	No	No
0.49	0.19	0.01	0.31	7.1	7.1	0.10	No
0.49	0.11	0.01	0.36	7.0	6.8	0.10	15

tallized as yellow needles (mp 200–201°) and was collected by suction filtration (Bradlow and Vanderwerf, 1951): λ_{max}^{HOH} 268 nm (ϵ 4140), λ_{min}^{HOH} 276 nm (ϵ 3660).

Irradiation

Apparatus and Light Measurements. Seven General Electric germicidal lamps (mainly 254 nm) were mounted in each irradiator, as reported previously (Wang, 1958).

Irradiation of N^1-Methylnicotinamide Salts (IIIa). EFFECTS OF BUFFER (pH 7, 1 mM PHOSPHATE BUFFER) AND ETHANOL. Portions (100 ml) of 1 mM solution of IIIa in distilled water were irradiated for 5 hr (32°) with a light intensity of 55 mW/ft^2 under the following conditions. The readings listed above were obtained by diluting the solution to 0.1 mM (Figure 1).

EFFECT OF ACETALDEHYDE. A 0.1 mM solution of IIIa was prepared, each 100 ml containing 0.01 ml of the buffer and 0.15 ml of ethanol. The solution was irradiated for 40 min (32°) under the following conditions. Since acetaldehyde is sensitive to ultraviolet irradiation, a reference solution without IIIa was prepared by simultaneous irradiation with the sample solution; this was used to obtain the readings listed below.

EFFECT OF OXYGEN. A 0.1 mM solution of IIIa was prepared as above without acetaldehyde. The solution was saturated with O_2 by bubbling O_2 for 8 hr. There was no apparent spectral change at 2-, 4-, 6-, or 8-hr intervals. Afterwards, the solution was irradiated in a manner described above, and the spectral increase at 325 nm was about 10–20% lower than that in the absence of oxygen. Another portion of the solution was bubbled with O_2 for 24 hr. After restoring the volume of the solution, there was no apparent change in the spectrum.

Irradiation of 1,4-Dihydro-1-methyl-4-oxonicotinamide (IX), 1,6-Dihydro-1-methyl-6-oxonicotinamide (X), Trigonelline (XI), Pyridinium Methyl Iodide (IIIb), and Nicotinonitrile Methyl Iodide (IIIc). A 0.1 mM solution of each of the above compounds was prepared, each containing 0.01 ml of the buffer and 0.15 ml of ethanol. These solutions were irradiated for 40 min at a light intensity of 55 mW/ft^2. The spectral changes produced by the irradiation of these compounds are described in Figure 2.

Isolation of 2-Oxo Derivative (VIa) from the Irradiation of N^1-Methylnicotinamide Salts (IIIa)

One liter of 1 mM solution of the salt containing 1 ml

OD 265 nm		OD 325 nm			
Initial	Final	Initial	Final	pH	Acetaldehyde (mM)
0.49	0.11	0.01	0.36	7	No
0.49		0.01	0.18	7	0.3 (excess)
0.49		0.01	0.18	7	0.1 (equiv)

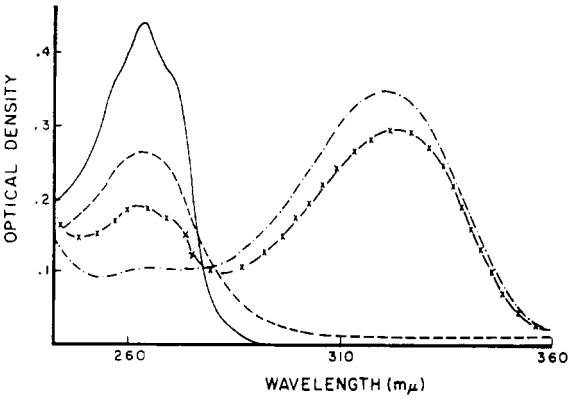

FIGURE 1: Ultraviolet absorption spectra of the N^1-methylnicotinamide salt before and after irradiation in various conditions. (——) Before irradiation, 1 mM; (– – –) irradiated for 8 hr in the absence of buffer; (X—X—) irradiated for 5 hr in the presence of buffer; (— · — · —) irradiated for 5 hr in the presence of buffer and alcohol.

of the pH 7 buffer and 13.5 ml of ethanol was irradiated for 4.5 hr. The irradiated solution was evaporated to dryness, and the residue was extracted several times with 10-ml portions of chloroform. The combined chloroform extracts were filtered through anhydrous sodium sulfate and were then evaporated to dryness. About 60 mg (40% theoretical yield) of crude product was obtained. This was twice recrystallized from acetone and melted at 223–224°. Its ultraviolet and infrared spectra were identical with the authentic sample prepared. No depression in mixture melting point was observed.

Anal. Calcd for $C_7H_8N_2O_2$: C, 55.25; H, 5.30; N, 18.41. Found: C, 55.43; H, 5.48; N, 18.64.

Results and Discussion

Effect of Buffer (Figure 1). Irradiation of IIIa in aqueous solution resulted in a general decrease of absorbancy in the ultraviolet region and appearance of a yellow color. After 8 hr, the ϵ_{max} decreased to *ca.* 50% and the pH was lowered from 5.5 to 3.3. However, at pH 7, the ϵ_{max} almost completely disappeared in 4–5 hr. This was accompanied by a simultaneous increase at *ca.* 330 nm which reached a maximum in this time interval. Apparently, the nature of the photoproducts varied with the pH values of the solutions.

Effect of Ethanol (Figure 1). Of particular interest is the observed absorbancy increase at *ca.* 330 nm, which resembled not only the spectral change associated with the reduction of NAD to NADH in biochemical reactions, but also the reported reduction of NAD and nicotinamide derivative by γ- or X-ray irradiation. In these cases, the reduction reactions were coupled with oxidation of ethanol to acetaldehyde. In this photoreaction, the presence of ethanol caused an apparent increase of 20%, both in yield and in rate of the 330-nm-absorbing material. However, the optimal concentration for ethanol was found to be 230–260 mole equiv in a 1 mM solution of IIIa. No apparent interpretation would account for this ethanol effect.

Isolation of 1,2-Dihydro-1-methyl-2-oxonicotinamide

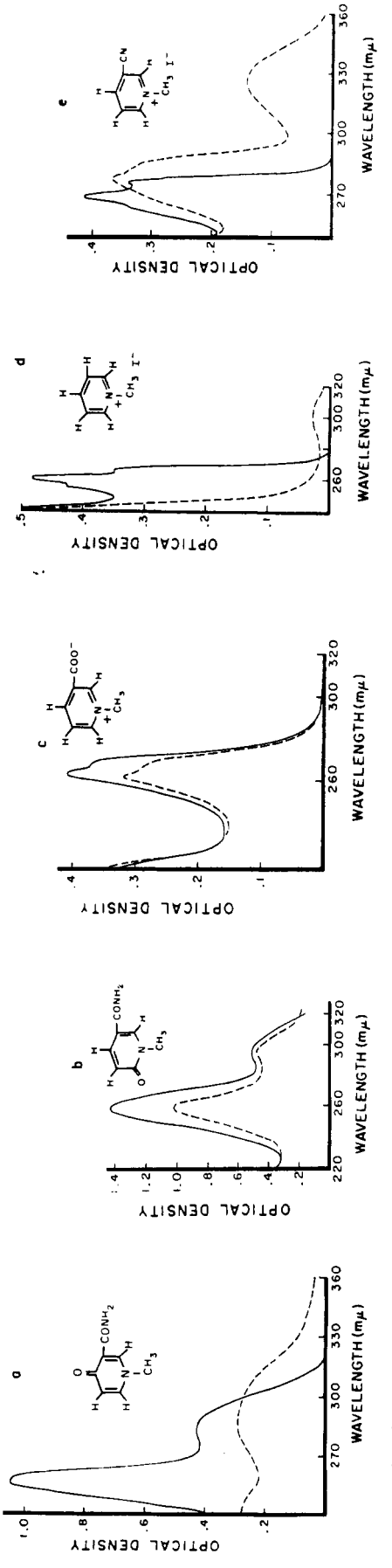

FIGURE 2: Ultraviolet absorption spectra before (——) and after (– – – –) irradiation of (a) 1, 4-dihydro-1-methyl-4-oxonicotinamide, (b) 1, 6-dihydro-1-methyl-6-oxonicotinamide, (c) trigoenelline, (d) pyridinium methyl iodide, and (e) nicotinonitrile methyl iodide.

(*VIa*). The photoproduct responsible for the absorbancy increase at *ca.* 330 nm was isolated and unequivocally identified as 1,2-dihydro-1-methyl-2-oxonicotinamide (VIa). Apparently, VIa is an oxidation rather than a reduction product. The reaction scheme IIIa–VIa (Scheme I) may account for the formation of the 2-oxo derivative. Based on the NAD chemistry (Kosower, 1960; Caughey and Schellenberg, 1966), the equilibria between III and V are mainly dependent upon the bond energy of C–X and the solvation energy of X^-. In this system, the formation of VIa from Va, a light step, causes a shift in equilibrium to the right. This would suggest that the dihydro intermediate (V) exists in buffered solutions.

Effect of Acetaldehyde or Oxygen. To determine whether this oxidation reaction could be facilitated by other oxidizing agents, acetaldehyde and oxygen were tested. The presence of either 1 mole equiv or an excess of acetaldehyde caused no apparent change either in rate or resultant absorbancy at *ca.* 330 nm. This suggests that an aldehyde has no effect on this photoreaction. Similarly, oxygen caused no apparent increase at 330-nm absorbancy; however, both the rate and the extent of the increase were slightly lower in the presence of oxygen.

Irradiation of 1,4-Dihydro-1-methyl-4-oxonicotinamide and 1,6-Dihydro-1-methyl-6-oxonicotinamide (IX and X, Figure 2a,b). Since 4-hydroxy-1,4-dihydro- or 6-hydroxy-1,6-dihydro derivatives (VII or VIII) may also exist as intermediates in the equilibrium mixture with V, they could react analogously to V, forming IX and X. These oxo compounds may further react during irradiation and thus elude detection by spectroscopy and/or isolation.

For these reasons, both 4- and 6-oxo derivatives were irradiated. The absorbancy of 4 isomer almost disappeared within 10 min, while the spectrum of 6 isomer decreased to 30% in 1 hr. This suggested that if X should form it would be detected, since the conversion of nicotinamide salt into 2-oxo compound is completed in 40 min. On the other hand, the more reactive 4 isomer would not be detected. These results, therefore, indicate that 6 isomer is neither an intermediate nor a product. However, the same conclusion could not be drawn with respect to 4-oxo derivatives, especially since the yield of 2-oxo compound obtained was only 50% according to the observed absorbancy increase at 330 nm.

Irradiation of Trigonelline (XI). Irradiation of trigonelline, a betaine or inner salt not readily forming a dihydro derivative, resulted only in a general decrease of absorbancy without the appearance of a new maximum between 220 and 360 nm (Figure 2c). This indicates that *N*-methyl-2-pyridone-3-carboxylic acid (XII) was not formed during irradiation, thereby substantiating the view that a dihydro derivative is ncecessary. (If decarboxylation should occur, the argument is still valid because it should result in IIIb or VIb (see below).)

Irradiation of Pyridinium Methyl Iodide (IIIb). To test whether a dihydro intermediate is the only requirement for the formation of this type of photooxidation product, IIIb was irradiated. Since only a general decrease in absorbancy was observed (Figure 2d) and no new maximum between 220 and 360 nm appeared, either *N*-methyl-2-pyridone (VIb) was not formed or it was unstable during irradiation. According to recent reports (Slomp *et al.*, 1961; Taylor and Kan, 1963), if VIb should form it could be detected spectroscopically. Thus, the presence of the amide group facilitates this photoreaction.

Irradiation of Nicotinonitrile Methyl Iodide (IIIc). Irradiation of IIIc under similar conditions caused a gradual increase of a new maximum at *ca.* 330 nm (Figure 2e), as in the irradiation of nicotinamide salts. This suggests that the presence of a CN and a $CONH_2$ group at C-3 has a similar effect on this reaction; however, this effect probably operates in the dark step (III–V). Further attempts are being made to understand these photoreactions.

Acknowledgments

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The Sequence at the 5′ Terminus of a Self-Replicating Variant of Viral Qβ Ribonucleic Acid*

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ABSTRACT: Nearest-neighbor analysis and resolution by DEAE column chromatography was used to identify the 5′-terminal sequence of a self-replicating variant derived from viral Qβ ribonucleic acid. The data indicate that the *plus* strand begins at the 5′ terminus with the octanucleotide $ppp(Gp)_4(Ap)_2(Cp)_2\ldots$ RNase T_1 digests of the appropriately labeled 5′ fragments confirms the sequence specified. The 5′ end of the complementary *minus* strand of the variant was shown to contain a much longer purine sequence starting with guanosine triphosphate. The 3′ end of the *minus* strand probably terminates with . . . GpGpUpUpCpCpCpC.

The availability of purified (Pace and Spiegelman, 1966a) Qβ replicase (Haruna and Spiegelman, 1965) which can mediate a virtually unlimited synthesis of biologically functional viral RNA (Spiegelman *et al.*, 1965) has made possible a variety of informative investigations into the mechanism of RNA replication. The use of a temperature-sensitive mutant led to the demonstration (Pace and Spiegelman, 1966b) that the RNA is the instructive agent in the replicative process. This finding rigorously justified the conclusion that the *in vitro* synthesis of a self-replicating molecule had been achieved.

Among the interesting possibilities thus generated was the feasibility of performing Darwinian experiments in which the molecules are exposed during replication to a variety of selection pressures. Mills *et al.* (1967) reported experiments designed to select molecules which could replicate faster than the original viral RNA. During the course of the serial transfers involved, variants of decreasing length made their appearance sequentially. The mutant isolated after the 74th transfer replicated some 15 times faster and contained only 550 of the 3600 residues present in the original Qβ-RNA. It has recently been shown (Levisohn and Spiegelman, 1968) that synthesis of copies by Qβ replicase can be initiated with a single strand of variant RNA. The clones produced provide the sort of uniformity required for chemical analysis of the RNA molecules and for sequence studies in particular.

A replicating molecule which has eliminated nonessential residues possesses evident advantages for a detailed examination of the replicative process. Of immediate interest is its obviously greater accessibility to sequence determination. Available methods for separating oligonucleotides (Tomlinson and Tener, 1963; Sanger *et al.*, 1965), combined with the advantages inherent in being able to synthesize chains with any one or all bases labeled, make it possible to obtain the sequence at the two ends of the molecules. Since the ends are probably involved in the initiation and termination of replication, information on the sequences at the termini should help to illuminate both the process of replication and the recognition mechanism which enables the replicase to distinguish one RNA molecule from another.

The present paper demonstrates that the 5′ end of the plus strand of a cloned Qβ-RNA variant begins with pppGpGpGpGpApApCpCp.[1]

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: GTP (pppG), guanosine triphosphate; pppGp, guanosine tetraphosphate; Ap, Gp, Cp, and Up are the 2′,3′-monophosphates of adenosine, guanosine, cytosine, and uridine, respectively. The term plus strand RNA refers to the variant RNA strands which have a sequence derived from Qβ viral RNA and *complementary* to the variant minus strand RNA which is synthesized during the replication process (Mills *et al.*, 1968). All double-stranded RNA forms, composed of plus and minus strands hydrogen bonded together, are termed HS RNA (Bishop *et al.*, 1967a).