Spectral and Biological Changes Induced in Nicotinic Acid and Related Compounds by Ultraviolet Light

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1. Irradiation of nicotinic acid, nicotinamide, nicotinamide *N*-oxide, *N*'-methyl-4-pyridone-3-carboxamide, reduced nicotinamide-adenine dinucleotide and pyridine with ultraviolet light at 253.7 m μ leads to striking spectral changes. 2. Nicotinic acid and nicotinamide are broken down to photosensitive intermediates which in turn undergo photodecomposition. 3. A major photoproduct of [7-¹⁴C]nicotinic acid is radioactive and absorbs ultraviolet light, but is inactive as a growth factor for *Candida pseudotropicalis*. 4. Irradiation of nicotinamide gives rise to small quantities of a biologically active photoproduct having the same R_F as nicotinic acid. A second photoproduct is also formed, but its identity has not yet been established. 5. Irradiation of nicotinamide *N*-oxide leads to the formation of several photoproducts, one of which has the same R_F as nicotinamide, absorbs ultraviolet light, and is biologically active. 6. Evidence is presented that irradiation of ethanolic solutions of *N*'-methyl-4-pyridone-3-carboxamide gives rise to acetaldehyde. 7. Irradiation of reduced nicotinamide-adenine dinucleotide in the presence of acetaldehyde leads to the formation of oxidized nicotinamide-adenine dinucleotide, which in turn can break down to nucleotide and/or nucleoside (depending on the conditions of the reaction). 8. The quantum yields of photolysis and the molar photosensitivities have been determined for *N*'-methyl-4-pyridone-3-carboxamide and nicotinamide *N*-oxide. 9. The possible biological significance of these photo-reactions is discussed in relation to photosynthesis, visual-pigment metabolism and ultraviolet-light-induced cell damage. 10. A four-step theory is presented for the biochemical evolution of oxidation-reduction systems, involving photoactivated transformations of pyridine derivatives.

We have shown that 4-PY,* a metabolite of nicotinamide in the rat, pig, monkey and man (Chang & Johnson, 1959, 1961, 1962), is highly sensitive to ultraviolet radiation, a fluorescent derivative being formed both on paper and in solution (Abelson & Boyle, 1963). These observations led us to undertake a more thorough investigation of the effects of ultraviolet light on nicotinic acid derivatives. First, a study was carried out of the spectral changes produced by irradiation. Second, the photoproducts of nicotinic acid and nicotinamide were studied by chromatography, radiochromatography and bioautography. Third, quantum yields were determined for 4-PY and nicotinamide *N*-oxide, the two most photosensitive compounds examined. It also seemed of interest to compare these values with published values for other biologically important compounds (Bücher &

Kaspers, 1947; Dartnall, 1957; Setlow, 1957; Rappoldt, Buisman & Havinga, 1958; Shugar, 1960; Schuberth, 1960; Holmström & Oster, 1961; Johns, Rapaport & Delbrück, 1962; Deering & Setlow, 1963; Levin, Setlow & Brown, 1964). Fourth, a general survey was made of the activity of nicotinic acid and related compounds as growth factors for *Candida pseudotropicalis*.

The participation of nicotinamide nucleotides as cofactors in photosynthesis and visual pigment metabolism suggests that they or their derivatives may also function as photocatalysts or photosensitizers in these biological systems. Such photoreactions might also have played a part in biochemical evolution.

METHODS

Assay of growth-factor activity. Individual compounds were assayed by the method of Johnson & Lin (1953), who added agar to the liquid medium described by Williams

* Abbreviation: 4-PY, *N*'-methyl-4-pyridone-3-carboxamide.

Table 1. *Biological activities of tryptophan and nicotinic acid derivatives*

The compounds were spotted on the centre of 2.5 cm. squares of chromatography paper, which were then placed for 10 min. on an agar surface containing *C. pseudotropicalis*. Growth in 48 hr. indicated by +; no growth, —.

Compound tested	Quantity ($\mu\text{g.}$)	Result	Confirming reference		
Nicotinic acid	0.1–20	+	*	†	‡
Nicotinamide	0.1–20	+	*	†	‡
NAD ⁺	100	+	*		‡
NADH	100	+			
NADP ⁺	100	+	*	†	
NADPH	100	+			
Trigonelline	50	+		†	‡
Nicotinuric acid	50	+	*	(†, —)	
Nicotinamide mononucleotide	50	+	*		
Quinolinic acid	50	—	*	†	
N'-Methyl-2-pyridone-5-carboxamide	20	—	*		
N'-Methyl-4-pyridone-3-carboxamide	20	—			
N'-Methyl-4-pyridone-3-carboxamide, <i>h</i> _v	20	—			
Nicotinamide N-oxide	50	—			
Nicotinamide N-oxide, <i>h</i> _v	50	+			
N'-Methylnicotinamide	50	—	*	†	
Tryptophan	50	—			‡
Formylkynurenine	50	—			
Kynurenine	50	—			‡
Kynurenic acid	50	—			
Xanthurenic acid	50	—			
3-Hydroxyanthranilic acid	50	—			‡
Anthranilic acid	50	—			
Pyridine	50 000	—			

* Johnson & Lin (1953).

† Williams (1946).

‡ Foley, Winter & Riley (1961).

(1946). The test micro-organism was the yeast *Candida pseudotropicalis* var. *lactosa* (N.R.R.L. no. Y-1204). Nicotinamide-deficient medium containing the test micro-organism was poured into Petri dishes and allowed to harden. The compounds to be tested were spotted on the centre of duplicate 2.5 cm. squares of chromatography paper. The squares were placed in contact with the agar surface for 10 min. and then removed. The Petri dishes were incubated for 24–48 hr. at room temperature (25°). Good growth was obtained with 1.0 $\mu\text{g.}$ of nicotinic acid or nicotinamide. The results of these experiments are shown in Table 1.

Conditions of irradiation. Irradiations were carried out with a 4.6 w Mineralight Handlamp, model SL2537 (Ultraviolet Products Inc., San Gabriel, Calif., U.S.A.), which delivers 70 $\mu\text{w/cm.}^2$ at 45 cm., of which 90% is at 253.7 m μ . Solutions were irradiated in quartz cuvettes which were in direct contact with the lamp filter. Under these conditions the lamp output, determined by ferric oxalate actinometry (Hatchard & Parker, 1956), was 47.9×10^{-8} mole-quanta/cm.²min. To allow for effects due to heat from the lamp, a control sample wrapped in metal foil was placed alongside the test solution throughout the irradiation period.

For deoxygenation, 3.0 ml. of the solution was pipetted into a cuvette, which was then placed in a beaker. The beaker was closed with a rubber bung provided with a nitrogen-inlet tube, which extended to the bottom of the cuvette. A small outlet tube was also present. After

nitrogen had been bubbled through the solution for 30 min. the inlet tube was raised and the cuvette stoppered. The stopper was so placed in the beaker that it could be inserted into the cuvette without opening the beaker. The stoppered cuvette was then removed from the beaker and irradiated as described in the preceding paragraph.

NADH was irradiated in water or buffers (50 mm. KH_2PO_4 or 50 mm. tris, adjusted to pH 7.5 with 5 N-NaOH), with or without acetaldehyde, and also in ethanol. The other compounds were irradiated in water or in various concentrations of ethanol in water. Spectra were determined at intervals in a Beckman DB recording spectrophotometer.

For irradiation in the dry state, 20 $\mu\text{g.}$ of each compound was spotted on Whatman no. 1 filter paper, and irradiated for 5 min. at 1.25 cm. The results of these experiments are summarized in Table 2.

Additional procedures for studying irradiation products of nicotinic acid and nicotinamide. (A) Irradiation, chromatography and ultraviolet photography. Nicotinic acid or nicotinamide (90 $\mu\text{g.}$) was irradiated in 3.0 ml. of 96% (v/v) ethanol for 30, 45 or 75 min. The irradiated solutions or portions thereof were concentrated, and chromatographed on methanol-washed Whatman no. 1 paper in butan-1-ol-aq. NH_3 (sp.gr. 0.88)–water (75:1:11, by vol.) together with an unirradiated control. After drying, the chromatograms were photographed according to the method of Abelson & Rosenfeld (1962), and then subjected to procedure (B) or (C).

Table 2. *Colour and fluorescence of some nicotinic acid derivatives after irradiation on paper*

A 20 μ g. sample of each compound was spotted on filter paper and irradiated for 5 min. at 253.7 $m\mu$.

Compound	Colour	Fluorescence in 360 $m\mu$ light	Remarks
Nicotinic acid	Faint yellow	Bluish white	
Nicotinamide	Faint yellow	Bluish white	
Trigonelline sulphate	Yellowish green	Greenish white	
N'-Methylnicotinamide	Pale yellow	Bluish white	
Nicotinuric acid	Nil	Faint bluish white	
N'-Methyl-2-pyridone-5-carboxamide	Nil	Faint dark blue	
N'-Methyl-4-pyridone-3-carboxamide	Nil	Bright blue, appears in a few seconds	Absorption decreased at 254 $m\mu$
Nicotinamide N-oxide	Faint yellow	Nil	Absorption decreased at 254 $m\mu$, and increased at 360 $m\mu$

(B) Radiochromatography. Before irradiation, 3×10^5 disintegrations/min. of $[7-^{14}C]$ nicotinic acid (1.66 mc/m-mole) or $[7-^{14}C]$ nicotinamide (6.56 mc/m-mole) were added to the solution containing the respective unlabelled compound. The radioactive compounds were purchased from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. After irradiation the entire 3 ml. was dried down and chromatographed overnight. The developed chromatograms were cut into strips 3.75 cm. wide, and the radioactive spots located with a chromatogram scanner (model ACSHI; Forro Scientific Co., Evanston, Ill., U.S.A.). Chromatograms of unirradiated $[7-^{14}C]$ nicotinic acid or $[7-^{14}C]$ nicotinamide, with (Figs. 2 and 5) or without added carrier, showed only a single radioactive peak. Radioactive contaminants, if any, amounted to no more than 3% of the total radioactivity.

(C) Bioautography. The general procedure was similar to that described above for the assay of individual compounds except that, to provide a large surface of contact with the chromatogram, the medium was poured into a 30 cm. $\times$ 45 cm. Pyrex tray fitted with a plate-glass cover (Johnson & Lin, 1953). Chromatography was carried out as in the preceding paragraph, except that 0.5 ml. portions of the irradiated solutions were taken for chromatography because of the extreme sensitivity of *C. pseudotropicalis* to nicotinic acid and nicotinamide, and the chromatograms were run for only 10.5 hr. to fit the Pyrex tray. After the medium had been exposed to a chromatogram for 10 min. and subsequently incubated for 48 hr., it was refrigerated until it was photographed.

Determination of quantum yield. Actinometry and quantum-yield determinations were done under identical conditions. Solutions of the substance being tested were irradiated in stoppered quartz spectrophotometer cuvettes, of 10 mm. light-path, which were placed in direct contact with the lamp filter. The concentrations were such that the initial extinction at the principal absorption maximum was between 0.75 and 0.80. The decrease in extinction at this wavelength was used for calculating the amount decomposed. For calculation of the quanta absorbed we used the extinction at 253.7 $m\mu$ rather than the wavelength of maximum absorption, because in none of the photo-reactions studied did the latter wavelength coincide with the maximum emission of the handlamp. Since the decrease in extinction at 253.7 $m\mu$ was linear with time for the first

0.5–1.0 min., the mean value before and after irradiation was used for calculating the total quanta absorbed. The quantum yield (ϕ) is given by the expression

$$\phi = \frac{(\Delta E/\epsilon) \times 10^{-3}}{I_0(1-T)t}$$

where $T = 10^{-E}$, ΔE is the decrease in extinction at the principal absorption maximum, ϵ is the molar extinction coefficient (ml. cm.⁻¹ m-mole⁻¹) at this wavelength, 10^{-3} is the factor converting m-moles/ml. to moles/ml., I_0 is the light-intensity (expressed in mole-quanta/cm.² min.), E' is the arithmetic mean of the extinction at 253.7 $m\mu$ before and after irradiation, and t is the time in min. In some experiments a quartz cuvette filled with 25% acetic acid was placed between the filter of the lamp and the sample cuvette, to cut off any light of wavelength below 240 $m\mu$ (Shugar, 1960). The light reaching the sample cuvette under these conditions was measured by ferric oxalate actinometry.

Detection of NAD⁺ after irradiation of NADH. The amount of NADH that had reacted was determined by the decrease in E_{340} . The reaction with yeast alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC 1.1.1.1; Sigma Chemical Co., St Louis, Mo., U.S.A.) was used to detect NAD⁺ in the irradiated samples. This method is specific for NAD⁺ with the exception of some synthetic analogues described by Kaplan (1961). The cyanide-addition reaction was used for the determination of NMN plus nicotinamide riboside (Colowick, Kaplan & Ciotti, 1951). Of the photo-products likely to be present in the reaction mixture only these two compounds and NAD⁺ give a cyanide reaction. The cyanide complexes of all three compounds have the same molar extinction coefficient at 326 $m\mu$.

Identification of acetaldehyde as an irradiation product of N'-methyl-4-pyridone-3-carboxamide in 1% ethanol. (a) By reaction with *p*-phenylphenol. A 3 ml. portion of 1% (v/v) ethanol containing 30 μ g. of 4-PY was irradiated in a stoppered quartz cuvette for 90 min. The volatile products of the reaction were carried over by a stream of nitrogen into H₂SO₄-CuSO₄ solution [6 ml. of conc. H₂SO₄, 0.9 ml. of water and 0.05 ml. of 4% (w/v) CuSO₄.5H₂O]. The acetaldehyde was determined by the modified Barker-Summerson procedure described by Swallow (1953).

(b) As the dinitrophenylhydrazone. The photoreaction

was carried out as described in the preceding paragraph except that the volatile products were absorbed in 0.5 ml. of 0.4 *N*-H₂SO₄ containing 1.25 mg. of 2,4-dinitrophenylhydrazine. The solution was then extracted with 3.0 ml. of chloroform, and the extract was chromatographed on methanol-washed Whatman no. 1 paper with hexane-methanol (24:1, v/v) as the mobile phase, and methanol-propylene glycol (4:1, v/v) as the stationary phase (Ellis, Gaddis & Currie, 1958).

(c) As the radioactive dinitrophenylhydrazone. The procedure was as in the preceding paragraph except that 5% of the total ethanol was [1-¹⁴C]ethanol (1.8 mc/m-mole). [1-¹⁴C]Ethanol was purchased from the New England Nuclear Corp., Boston, Mass., U.S.A. If one molecule of 4-PY reacted with one molecule of ethanol, the final activity of [1-¹⁴C]acetaldehyde would be 0.019 μ C, which is sufficient for radioautography. After 90 min. irradiation the combined products of two reactions were absorbed in 1.0 ml. of the 2,4-dinitrophenylhydrazine reagent. The reaction products were then extracted with 3.0 ml. of hexane. (Hexane is a selective reagent for the extraction of acetaldehyde 2,4-dinitrophenylhydrazone, leaving nearly all the excess of 2,4-dinitrophenylhydrazine in the acid phase. Chloroform, however, extracted both compounds.) Carrier acetaldehyde 2,4-dinitrophenylhydrazone (2–5 mg.) was added to the hexane extract and paper chromatography was carried out as in the preceding paragraph. The chromatogram was exposed to Kodak No-Screen X-ray film for 5 days.

RESULTS

Nicotinic acid

Biological activity. Nicotinic acid supported the growth of *C. pseudotropicalis* in amounts as low as 0.1 μ g. (Table 1). When 90 μ g. of nicotinic acid in 3.0 ml. of 96% ethanol was irradiated at 253.7 m μ for 150 min., biological activity was completely lost.

Spectral changes. When nicotinic acid was irradiated in 96% ethanol for 15 min. there was an increase in the absorption maximum at 262 m μ , and a new maximum appeared at 333 m μ (Fig. 1). After an additional 60 min. irradiation the short wavelength maximum was displaced to 272 m μ and the 333 m μ peak disappeared. Further irradiation caused a decrease in the 272 m μ peak. Fig. 1 shows no isosbestic point for the overall photoreaction. Nicotinic acid in water showed little spectral change: irradiation for 100 min. resulted mainly in a decrease in the absorption maximum (262 m μ) of less than 15% of the initial extinction (0.8).

Irradiation, chromatography and ultraviolet photography. When nicotinic acid was irradiated on paper, a bluish-white fluorescence developed. When solutions of nicotinic acid were irradiated and subsequently chromatographed (procedure A, with the entire sample), inspection and photography of the chromatogram (CR-1) in 253.7 m μ light showed an ultraviolet-absorbing photoproduct (compound X) with *R_F* 0.26, migrating just behind

Fig. 1. Ultraviolet-absorption spectra of nicotinic acid (60 μ g./3 ml. of 96% ethanol): —, before irradiation; ---, after 15 min. irradiation at 253.7 m μ ; - · - · -, after 75 min.; · · · ·, after 315 min.

Fig. 2. Radiochromatographs of nicotinic acid (90 μ g./3 ml. of 96% ethanol) + [7-¹⁴C]nicotinic acid (3×10^5 disintegrations/min.): (a) before irradiation; (b) after 30 min. irradiation at 253.7 m μ ; (c) after 45 min.; (d) after 75 min. Chromatography system: butan-1-ol-aq. NH₃ (sp.gr. 0.88)–water (75:1:11, by vol.). O, Origin; F, solvent front; X, compound X.

nicotinic acid (*R_F* 0.30). This photoproduct was present after 30, 45 and 75 min. irradiation. After 45 min., compound X and nicotinic acid appeared

to be present in nearly equal amounts. The chromatogram of this sample was therefore chosen for further study. The R_F 0.18–0.36 region was cut out, and rechromatographed for 41 hr. in the same solvent system. A photograph of this second chromatogram (CR-2) showed that compound X was now completely resolved from unchanged nicotinic acid. In addition to compound X, chromatograms of irradiated samples showed fluorescence at the origin and streaks along the chromatogram, CR-1.

Radiochromatography. Fig. 2 shows the distribution of radioactivity before and after irradiation (procedure B, CR-1). The R_F of the initial single peak was 0.30. Irradiation for 30 min. produced compound X (R_F 0.26), as well as some slowly migrating material that remained near the origin. The amount of radioactive material with R_F 0.30 decreased considerably as the irradiation time was increased until, after 75 min., only about 10% of

the original radioactivity remained in the nicotinic acid region. Radiochromatography of CR-2 showed that compound X and nicotinic acid were completely separated.

Bioautography. Fig. 3 shows that irradiation led to a gradual loss of biological activity, yet after 75 min. some still remained (procedure C). However, compound X and residual nicotinic acid were not resolved in this bioautogram and we could not therefore assess the biological activity of compound X. For this reason, the two ultraviolet-absorbing spots on CR-2 were cut out and assayed separately. Compound X was inactive.

Nicotinamide

Biological activity. The behaviour of this compound was very similar to that of nicotinic acid. Yeast colonies were detectable even with amounts as low as 0.1 μg . (Table 1); but when 90 μg . was irradiated in 3.0 ml. of 96% ethanol for 150 min. it no longer supported colony growth.

Spectral changes. The changes observed were also similar to those obtained on irradiation of nicotinic acid, i.e. in 96% ethanol there was an increase in the absorption maximum at 262 $m\mu$ and a new maximum at 335 $m\mu$ (Fig. 4). Irradiation for an additional 60 min. caused a bathochromic shift from 262 $m\mu$ to 270 $m\mu$, and a decrease in the 335 $m\mu$ peak. Further irradiation caused a decrease in the 270 $m\mu$ peak, which now shifted to 275 $m\mu$. The

Fig. 3. Dark-field photograph of a bioautogram of nicotinic acid (90 μg ./3 ml. of 96% ethanol) irradiated at 253.7 $m\mu$; 15 μg . portions were used in the assay. The chromatography system was butan-1-ol-aq. NH_3 (sp.gr. 0.88)–water (75:1:11, by vol.). The developed chromatogram was transferred to an agar surface containing *C. pseudotropicalis*. The origin is at the bottom of the photograph, the solvent front at the top.

Fig. 4. Ultraviolet-absorption spectra of nicotinamide (60 μg ./3 ml. of 96% ethanol): —, before irradiation; ----, after 15 min. irradiation at 253.7 $m\mu$; ·····, after 315 min.

335 $m\mu$ peak completely disappeared. Fig. 4 shows no isosbestic point for the overall reaction. Nicotinamide in water showed no notable spectral changes after 100 min. irradiation.

Irradiation, chromatography and ultraviolet photography. When nicotinamide was irradiated on paper it developed a bluish-white fluorescence. When solutions of nicotinamide were irradiated and subsequently chromatographed (procedure A, entire sample), inspection and photography of the chromatogram in 253.7 $m\mu$ light showed an ultraviolet-absorbing photoproduct (compound Y) with R_F 0.65 (compared with 0.70 for unirradiated nicotinamide), which appeared within 30 min. In addition, fluorescence was found near the origin.

Radiochromatography. Fig. 5 summarizes the distribution of radioactivity before and after irradiation (procedure B). The R_F of the initial peak was 0.70. Irradiation produced material that remained at the origin, as well as photo-products that streaked along the chromatogram from the origin almost to the solvent front. As the irradiation time was increased the radioactivity due to nicotinamide decreased, until at 75 min. it accounted for less than half the total radioactivity. At the same time there was a gradual increase in the radioactivity both at the origin and along the chromatogram. No discrete radioactive peak for compound Y could be seen at any of the irradiation times.

Bioautography. Chromatography (procedure C) of unirradiated nicotinamide produced only one region with growth-factor activity. After irradiation, however, traces of a biologically active photoproduct (compound Z) were formed with the

same R_F as authentic nicotinic acid (0.3). The amounts of compound Z were too small to be detected in the radiochromatographs (Fig. 5) or ultraviolet photographs. The results of a typical experiment are shown in Fig. 6.

Nicotinamide N-oxide

Biological activity. This compound was inactive (Table 1). However, irradiation of 100 μg . in 3.0 ml. of 96% ethanol for 30 min. led to the formation of an active photoproduct.

Spectral changes. The absorption spectrum was characterized by a single maximum at 260 $m\mu$ in water, or 270 $m\mu$ in 96% ethanol (Fig. 7). After irradiation in either solution the 260–270 $m\mu$ peak disappeared, simultaneously with the formation of two new peaks, at 314 $m\mu$ and 360 $m\mu$. Further irradiation caused a rapid decrease in the 314 $m\mu$ peak, and a slow decrease at 360 $m\mu$. Fig. 7 shows no isosbestic point for the overall reaction. The

Fig. 5. Radiochromatographs of nicotinamide + [7- ^{14}C]-nicotinamide (3×10^5 disintegrations/min.). Irradiation conditions, concentrations, chromatography system and symbols were as given in Fig. 2.

Fig. 6. Dark-field photograph of a bioautogram of nicotinamide. Irradiation conditions, concentrations, and bioassay were as given in Fig. 3. A nicotinic acid standard ($1 \mu g$) was added to unirradiated nicotinamide on the left. The origin is at the bottom of the photograph, the solvent front at the top.

Fig. 7. Ultraviolet-absorption spectra of nicotinamide *N*-oxide (30 µg./3 ml. of 96% ethanol): —, before irradiation; ---, after 5 min. irradiation at 253.7 mµ; ·····, after 30 min.

quantum yield of photolysis of nicotinamide *N*-oxide was 35×10^{-3} in water and 62×10^{-3} in 96% ethanol. The quantum yield increased continuously as the ethanol concentration was increased from 0 to 96% (Fig. 8).

Irradiation, chromatography and ultraviolet photography. The behaviour of nicotinamide *N*-oxide on paper was analogous to that in solution, in that irradiation led to disappearance of absorption at short wavelengths, with the simultaneous development of absorption at 360 mµ. This very unusual behaviour has proved useful for identification purposes. To study the photoproducts, chromatograms were prepared according to procedure A (by using the entire sample of 100 µg., and irradiation times of 5, 30 and 60 min.). Standards of nicotinic acid and nicotinamide (10 µg. each) were added to unirradiated nicotinamide *N*-oxide before chromatography. Inspection and photography of the chromatograms in 253.7 mµ light showed that nicotinamide *N*-oxide had R_F 0.35. After 5–60 min. irradiation an additional ultraviolet-absorbing spot appeared, with the same R_F (0.74) as authentic nicotinamide. In addition, several other absorbing spots and some fluorescing spots were seen.

Bioautography. Further evidence for the identity of nicotinamide as an irradiation product of nicotinamide *N*-oxide was provided by a bioautogram prepared from the chromatogram described in the preceding paragraph. This showed only one active photoproduct, with the same R_F as nicotin-

Fig. 8. Quantum yields as a function of ethanol concentration in aqueous solution: O, nicotinamide *N*-oxide (24–30 µg./3 ml.); ●, *N'*-methyl-4-pyridone-3-carboxamide (30 µg./3 ml.).

amide (0.74). The yeast colony sizes suggested that about 5 µg. was present after 5 and 30 min. irradiation and about 2 µg. after 60 min. irradiation. The absence of spectral evidence for nicotinamide as a photoproduct of nicotinamide *N*-oxide (Fig. 7) is not surprising, because in these experiments the expected yield (0.5 µg.) would contribute no more than 0.03 to the extinction of irradiated nicotinamide *N*-oxide at 262 mµ.

NADH and NADPH

NADH supported the growth of *C. pseudotropicalis* (Table 1). Irradiation of an aqueous solution at pH 7.5 caused a decrease in extinction at 340 mµ (Fig. 9). After 70 min. there was no extinction at 340 mµ. This effect was inhibited by ethanol, but in the presence of 1% (v/v) acetaldehyde the decrease in absorption was markedly accelerated (Fig. 10). Further studies were carried out to determine whether NAD^+ was formed by irradiation in the presence of 1% acetaldehyde. In phosphate buffer 25% of the photoproducts was NAD^+ and 35% NMN plus nicotinamide riboside. However, in water or tris buffer 60–70% of the photoproducts was NAD^+ , and no significant amount of NMN or nicotinamide riboside was found. The rate of photodecomposition was faster in phosphate buffer than in water or tris buffer. NADPH also supported

Fig. 9. Ultraviolet-absorption spectra of NADH (90 μ g./3 ml. of 50 mM-phosphate buffer, pH 7.5): —, before irradiation; ----, after 10 min. irradiation at 253.7 m μ .

the growth of *C. pseudotropicalis* (Table 1). Preliminary experiments showed that the effects of irradiation were similar to those obtained with NADH.

Irradiation of N'-methyl-4-pyridone-3-carboxamide as an enzyme model system

Our observations on the effects of radiation on the absorption and fluorescence spectra of this compound have already been reported (Abelson & Boyle, 1963). We showed that irradiation in 96% ethanol abolished the maximum at 258 m μ and caused a bathochromic shift in the 285 m μ maximum. These spectral changes were seen at concentrations of ethanol as low as 1%. The effect was also manifested in various buffers over a wide pH range. In the absence of oxygen the rate of photodecomposition was unchanged. The quantum yield as a function of the ethanol concentration is shown in Fig. 8. In the absence of ethanol ϕ was 1.8×10^{-3} but a maximum value of 39×10^{-3} was obtained in 40% (v/v) ethanol. These results contrast with those obtained with nicotinamide *N*-oxide, which showed a continuous increase in the quantum yield with increasing ethanol concentration.

Since ethanol is oxidized in the presence of nicotinamide nucleotides by alcohol dehydrogenase, γ -rays (Swallow, 1953) and X-rays (Barron, Johnson & Cobure, 1954), tests were made for the formation of acetaldehyde when ethanol and 4-PY were irradiated. When *p*-phenylphenol was added to the trapped volatile reaction products, the typical blue colour ($\lambda_{\max}$ 570 m μ) given by acetaldehyde was seen. Irradiation of ethanol alone, or of 4-PY

Fig. 10. Ultraviolet-absorption spectra of NADH (90 μ g./3 ml. of 50 mM-phosphate buffer, pH 7.5) + 1% acetaldehyde: —, before irradiation; ----, after 10 min. irradiation at 253.7 m μ ; read against blank containing 1% acetaldehyde in buffer.

in the absence of ethanol, resulted in only a faint colour. When the volatile products were led into 2,4-dinitrophenylhydrazine solution crystals formed, but no crystals were formed if the irradiation step was omitted. Paper chromatography of the hydrazone produced a major and a minor spot which absorbed ultraviolet light at 360 m μ . The R_F values were identical with those obtained with authentic acetaldehyde 2,4-dinitrophenylhydrazone. These experiments with 2,4-dinitrophenylhydrazine were repeated with [1- 14 C]ethanol. The radioactivity migrated with the major carrier spot. When the [1- 14 C]ethanol was irradiated without 4-PY there was no radioactivity associated with the carrier spots.

Nicotinuric acid

This compound was biologically active (Table 1). When it was irradiated in 96% ethanol there was an increase in the absorption maximum which was similar to, but less pronounced than, the increase observed when nicotinic acid and nicotinamide were irradiated (Fig. 11). However, unlike nicotinic acid or nicotinamide, no new peak appeared at 335 m μ . Irradiation of nicotinuric acid in water led to a decrease in the absorption maximum (Fig. 12), whereas irradiation on paper gave rise to a faint bluish-white fluorescence.

Fig. 11. Ultraviolet-absorption spectra of nicotinuric acid ($60 \mu\text{g./3 ml.}$ of 96% ethanol): —, before irradiation; ----, after 30 min. irradiation at $253.7 \text{ m}\mu$.

Fig. 13. Ultraviolet-absorption spectra of nicotinamide mononucleotide ($180 \mu\text{g./3 ml.}$ of 2% ethanol): —, before irradiation; ----, after 30 min. irradiation at $253.7 \text{ m}\mu$.

Fig. 12. Ultraviolet-absorption spectra of nicotinuric acid ($60 \mu\text{g./3 ml.}$ of water): —, before irradiation; ----, after 30 min. irradiation at $253.7 \text{ m}\mu$.

Nicotinamide mononucleotide

This compound also showed growth-factor activity (Table 1). After irradiation in 2% (v/v) ethanol there was a slight decrease in the absorption maximum at $265 \text{ m}\mu$ (Fig. 13).

Pyridine

This compound had no growth-factor activity (Table 1). When irradiated in water, very strong absorption developed at $360 \text{ m}\mu$ (Fig. 14). This

Fig. 14. Ultraviolet-absorption spectra of pyridine ($12 \mu\text{g./3 ml.}$ of water): —, before irradiation; ----, after 4 min. irradiation at $253.7 \text{ m}\mu$; ·····, same + 20 hr. at room temp. (25°).

change was partially reversed when the solution was left standing overnight. Irradiation in ethanol produced similar but less profound spectral changes. Freytag & Neudert (1932) described the production of a yellow colour when pyridine was irradiated on paper. Subsequent exposure to daylight reconstituted the pyridine. This reversible change was

ascribed to ring rupture (Freytag, 1948), but no precise spectral data were published.

Other compounds

Biological activities are summarized in Table 1. Little or no spectral change was observed when aqueous or ethanolic solutions of trigonelline sulphate, *N*'-methylnicotinamide, *N*'-methyl-2-pyridone-5-carboxamide, NAD⁺ or NADP⁺ were irradiated. The results of irradiation on paper are given in Table 2.

DISCUSSION

Most of the substances tested showed spectral changes when irradiated in solution. Nicotinamide *N*-oxide and 4-PY were the most photosensitive. Behaviour on paper was, in general, analogous to that in solution. Often a yellow colour developed, usually with fluorescence, and with nicotinamide *N*-oxide the short-wave absorption disappeared and was replaced by long-wave absorption. These properties can be utilized as spot tests, which are quite specific for 4-PY and nicotinamide *N*-oxide, but are not very sensitive, except with 4-PY.

The ultraviolet-absorption spectra suggest that nicotinic acid forms a photoproduct (or photoproducts) that in turn undergoes further photoreaction. The ultraviolet photographs and radiochromatographs show that one of the photoproducts, which fluoresced in 253.7 m μ light, migrated very slowly in our solvent system. The total amount of this photoproduct remained about the same between 30 and 75 min. irradiation. Another photoproduct (compound X) migrated just behind nicotinic acid, and, like nicotinic acid, absorbed 253.7 m μ light. The total amount of this photoproduct also remained about the same between 30 and 75 min. Comparison of the radiochromatograph for unirradiated nicotinic acid with that of nicotinic acid after 75 min. irradiation showed that approximately a third of the total radioactivity had disappeared. The observation that the total amount of radioactivity due to the two photo-intermediates remained unchanged, in contrast with the progressive decrease in nicotinic acid, suggests a sequence of photoreactions in which [7-¹⁴C]nicotinic acid gave rise to radioactive intermediates that were sequentially broken down to form ¹⁴CO₂ and unlabelled derivatives.

The spectral data for irradiated nicotinamide indicate that photosensitive intermediates were also formed, and that they decomposed as the irradiation time was increased. Ultraviolet photographs and radiochromatographs show that one of these photoproducts (which also fluoresced in 253.7 m μ light) migrated so slowly in our solvent

system that much of it remained at the origin. However, unlike the nicotinic acid photoproducts, the amount of this photoproduct increased as the irradiation time was increased. Trace amounts (too small to be seen in Fig. 5) of two other photoproducts were also formed. One (compound Y), migrating just behind nicotinamide, showed up on the ultraviolet photographs; the other (compound Z) was detected by bioautography, and had *R_f* identical with that of nicotinic acid. Fig. 6 indicates that if this photoproduct was nicotinic acid, then it was present in a steady-state quantity of no more than 3 μ g. (0.5 μ g. $\times$ 6). When the radiochromatograph of unirradiated nicotinamide was compared with that of nicotinamide irradiated for 75 min., it appears that little, if any, of the total radioactivity had been lost. This suggests that the sequence of photoreactions in which nicotinamide was transformed into a nicotinic acid-like intermediate, that in turn decomposed to form carbon dioxide and unlabelled derivatives, was only a side reaction. This conclusion is also supported by the low concentration of the intermediate of this sequence. The photoreaction in which nicotinamide is formed from nicotinamide *N*-oxide is similar to the photoreaction recently reported by Levin *et al.* (1964) in which adenine is formed from adenine *N*¹-oxide. The loss of biological activity when a compound is exposed to ultraviolet irradiation is not surprising, but the formation of a biologically active photoproduct from an inactive precursor is unusual, and recalls the formation of vitamin D by irradiation of ergosterol.

Nicotinic acid, nicotinamide and nicotinamide *N*-oxide were investigated spectrophotometrically after irradiation for different periods of time. The fact that none of the resulting families of curves has an isosbestic point is further evidence that each of these photoreactions leads to the formation of several products.

The effect of ultraviolet light on NADH, namely conversion into NAD⁺, appears to be similar to the effect of X-rays (Barron *et al.*, 1954; Myers, 1960). The ultraviolet-induced oxidation, which is accelerated by acetaldehyde, simulates the dehydrogenase-catalysed reaction between NADH and acetaldehyde. Frisell & Mackenzie (1959) have reported photocatalytic oxidation of NADH when pyruvate or riboflavine phosphate was present, but observed no reaction with NADH alone. However, these workers used excitation in the region of 350 m μ , which we have found to be much less effective than excitation at 253.7 m μ . Moreover, their dithionite reduction of the photoproducts is less specific for NAD⁺ than the enzyme assay (Ekert & Monier, 1958).

In some respects the effects of ultraviolet light on NAD⁺ also resemble those of X-rays. Thus in

the presence of air neither X-rays (Barron *et al.* 1954; Myers, 1960) nor ultraviolet radiation (Seraydarian, Cohen & Sable, 1954; Carter, 1950; Ekert & Monier, 1958) produce significant change in the absorption spectrum unless very high doses are used, in which case there is a fall in E_{260} (Carter, 1950; Barron *et al.* 1954; Myers, 1960). Despite their negligible effect on the absorption spectrum, both X-rays (Myers, 1960) and ultraviolet radiation (Seraydarian *et al.* 1954; Seraydarian, 1956; Ekert & Monier, 1958) rapidly destroy coenzyme activity. Since adenine, AMP and ADP appear to be major photo-products, it has been concluded that the loss of coenzyme activity is due to bond cleavage (Carter, 1950; Seraydarian *et al.* 1954; Ekert & Monier, 1958). Seraydarian *et al.* (1954) suggested that the persistence of absorption at 260 m μ is due to the presence of intact adenine in the photoproducts. Barron *et al.* (1954) and Myers (1960) also considered intact adenine fragments to be responsible for their observations that the absorption spectrum of NAD⁺ was unaffected by X-rays, although they did not study the radiation products. Under other conditions, the effects of the two forms of radiation on NAD⁺ are different. Thus Barron *et al.* (1954) and Stein & Swallow (1958) found that anaerobic exposure of NAD⁺ to X-rays in the presence of 0.5 M-ethanol produced an absorption maximum near 340 m μ . Since the product was enzymically inactive it was concluded that a dimer had been formed (Stein & Swallow, 1958). Under similar anaerobic conditions we found that ultraviolet irradiation of NAD⁺ produces no changes in the absorption spectrum.

In spite of the superficial similarity between the NAD⁺-linked oxidation of ethanol by alcohol dehydrogenase and the 4-pyridone-linked oxidation of ethanol by ultraviolet light (a resemblance strengthened by the reactivity of the *para*-position in both compounds), 4-PY exhibited no coenzyme activity in the alcohol-dehydrogenase reaction (neither did it inhibit the action of NAD⁺). Conversely NAD⁺ and NADP⁺ failed to oxidize ethanol when irradiated with ultraviolet light, whether or not oxygen was excluded. The fact that oxygen did not affect the rate of photodecomposition of 4-PY excludes oxygen as an essential participant in the reaction in which acetaldehyde is formed. Also, ethanol cannot function as the primary receptor of radiation because (a) it does not absorb light at 253.7 m μ , and (b) the quantum yield does not change when light below 240 m μ is filtered out. We conclude that the photocatalytic process is initiated by a primary excited state of the 4-PY molecule itself.

The photoreactions described above may be summarized as follows (identification of compounds marked with asterisks is tentative):

The quantum yield for purines and pyrimidines ranges from 0.06×10^{-3} for adenine to 21.6×10^{-3} for uridine (see Table 3). Thus the values for nicotinamide *N*-oxide of 62×10^{-3} in 96% ethanol and 35×10^{-3} in water can be considered very high. These values are of the same order of magnitude as that reported for adenine *N*¹-oxide, namely 100×10^{-3} (Levin *et al.* 1964). Brown, Levin & Murphy (1964) reported that adenosine *N*¹-oxide, 6 methyl-purine *N*¹-oxide and isoguanine *N*¹-oxide are also very sensitive to 253.7 m μ light. The quantum yield of 39×10^{-3} for 4-PY in 40% ethanol is also high in comparison with the values for purine and pyrimidine: in addition, the yield is greater than that observed in either water or 96% ethanol. Since there is a more than 20-fold difference between the quantum yields in water and in 40% ethanol, it appears that the photoreaction of 4-PY in aqueous ethanol is a complex one involving two competing reactions. Of these, one is suppressed in water and the other in 96% ethanol. Our data show that one of these reactions involves the oxidation of ethanol to acetaldehyde. The other reaction may involve the addition of water to one of the double bonds of 4-PY, in a manner analogous to the photoreactions of pyrimidines (Shugar, 1960).

The quantum yield (ϕ) and the molar extinction coefficient (ϵ) are both indicative of the sensitivity of a compound to light. Indeed, Goodeve & Wood (1938) suggested the term 'photosensitivity' for the product ($\alpha\phi$) of the molecular extinction coefficient $\alpha = [(2.303 \times 10^{-3}/6.025 \times 10^{23}) \times \epsilon]$ and the quantum yield. They cite cases in which, even though one of these values was low, the rate of photodecomposition was rapid since the other value was high. Therefore, in comparisons of photocatalysed reactions, this product would be analogous to a rate constant. Dartnall (1957) converted the molecular photosensitivity into molar photosensitivity ($\epsilon\phi$), because of the widespread use of the molar extinction coefficient. Other names have been suggested for the product $\alpha\phi$, such as 'inactivation cross section' (Setlow, 1957), 'sensitivity' (Deering & Setlow, 1963) and 'decomposition cross section' (Levin *et al.* 1964). In all three publications the same symbol, σ , is used ($\sigma = \alpha\phi$). Johns *et al.* (1962) suggested another

Table 3. *Quantum yields and photosensitivities*

Substance	Solvent	$10^3 \times$ Quantum yield (ϕ)	ϵ	λ	Photosen- sitivity ($\epsilon\phi$)	Reference
<i>N'</i> -Methyl-4-pyridone- 3-carboxamide	Water	1.8	11 000	258	20	This paper
<i>N'</i> -Methyl-4-pyridone- 3-carboxamide	40% Ethanol	39	11 000	258	430	This paper
<i>N'</i> -Methyl-4-pyridone- 3-carboxamide	96% Ethanol	7.9	11 000	258	87	This paper
Nicotinamide <i>N</i> -oxide	Water	35	9 050	260	316	This paper
Nicotinamide <i>N</i> -oxide	96% Ethanol	62	10 250	270	635	This paper
Adenine	Water	0.06	13 800	260	0.8	Shugar (1960)
Uridine	Water	21.6	9 660	262	209	Shugar (1960)
Thymine	Water	0.4	7 653	264	3	Shugar (1960)
Thymine dinucleotide	Phosphate buffer	10	18 600	265	186	Deering & Setlow (1963) Johns <i>et al.</i> (1962)
Adenine <i>N</i> ¹ -oxide	Water	100	40 000	230	4000	Levin <i>et al.</i> (1964)
Cystine	Ethanol	20				Setlow (1957)
Previtamin D		400				Rappoldt <i>et al.</i> (1958)
Riboflavine	Water	6	12 500	445	75	Holmström & Oster (1961)
Visual purple	Aqueous	500	48 000–54 000		24 000–27 000	Dartnall (1957)
Myoglobin–CO	Phosphate and borate buffers	500–1000	60 000*	405	30 000–60 000	Bücher & Kaspers (1947) Schuberth (1960)

* Calculated from the authors' value for the absolute extinction coefficient, β ($\epsilon \times 2.303 \times 10^3$).

constant, the activity coefficient ($a = 2.303 \times 10^3 \times \epsilon\phi$). To emphasize the usefulness of the concept of photosensitivity, the molar photosensitivities of the compounds we are comparing are included in Table 3. These range from 0.8 for adenine to 30 000–60 000 for the myoglobin–carbon monoxide complex.

Photocatalysis of oxidations and reductions involving nicotinic acid derivatives and nicotinamide nucleotides has several implications.

First, such reactions provide a convenient model system showing many features analogous to enzymic transformations.

Second, the ease with which these effects can be demonstrated *in vitro* suggests the need for re-evaluating the function of pyridinium derivatives as direct receptors of radiation in photobiological processes. Nicotinamide nucleotide-linked redox systems play an important part in two photocatalytic pathways, visual-pigment metabolism and photosynthesis. The photochemical action of visible light in the bleaching of rhodopsin and its photocatalytic effect on *trans*-retinene isomerization have been fully documented. Chance (1964) suggested that a second mechanism may operate in the aphakic eye, and in certain insect visual systems which are responsive to ultraviolet radiation. He postulated that in these systems light-energy is absorbed by reduced nicotinamide nucleotides and transformed into fluorescent energy, which is subsequently transferred to other

compounds. We think that, in visual systems in general, at least two other possibilities deserve consideration. (1) In ultraviolet-sensitive systems direct photocatalysed reductions might well occur with NADH, as Frisell & Mackenzie (1959) have demonstrated by using pyruvate or riboflavine as hydrogen acceptor and a 350 m μ source. (2) In systems responsive to light of longer wavelengths photosensitized reactions might take place that are analogous to the light-induced oxidation of NADH by chlorophyll, in which the chlorophyll functions both as sensitizer and oxidant (Vernon, 1961).

Third, the damage inflicted on cells by ultraviolet light may in some cases be attributed, at least in part, to phototransformations in pyridine derivatives. The occurrence of absorption maxima near 260 m μ in both nucleic acid and pyridine derivatives suggests that evidence derived from action spectra alone cannot be regarded as diagnostic of nucleic acid damage, except in tissues where these compounds predominate. Indeed, Shugar (1960) and Setlow (1957) mention this possible role of free nucleotides and nucleotide coenzymes.

A role of photoactivated transformations of pyridine derivatives in organic evolution has been postulated by Frisell & Mackenzie (1959). We suggest that the following stages may have occurred in the evolution of oxidation–reduction systems. (1) A stage of simple synthesis, culminating in the

production of ultraviolet-absorbing compounds, particularly ultraviolet-sensitive hydrogen carriers such as pyridine derivatives. Ultraviolet-absorbing compounds have been detected in electric discharges by Oró (1963). (2) A stage of photocatalysed oxidations and reductions. This stage would terminate with the advent of atmospheric ozone, and subsequent exclusion of solar ultraviolet radiation. Steinman, Lemmon & Calvin (1964) have described photocatalysed polymerizations of glucose and of amino acids in the presence of the cyanamide dimer. If such reactions were a part of biological evolution, this also would be the stage of their activity. (3) A stage of photosensitized oxidations and reductions, utilizing visible light and sensitizers, such as chlorophyll (Vernon, 1961), synthesized in stage (2). (4) A stage of enzyme-catalysed oxidations and reductions, with enzyme proteins manufactured during stage (3). This scheme accords well with current views on the sequence of pre-biotic evolution as set out by Gaffron (1962).

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