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Gamma Radiolysis of Flavin Mononucleotide and Flavin-Adenine Dinucleotide in Aqueous Solution

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Flavin-adenine dinucleotide (FAD) and flavin mononucleotide (FMN) were irradiated with ^{60}Co γ-ray and G-values for their decomposition under aerated, argon and nitrous oxide saturated conditions at pH 6.5 were determined by spectrophotometric, fluorescence and HPLC measurements. Among the water-borne radicals the hydroxyl radical (OH) was found to be responsible for causing permanent changes in FAD and FMN. The striking similarity between the difference spectra of irradiated FAD and FMN in N_2O saturated conditions shows that the OH radicals attack at the isoalloxazine moiety. A plausible mechanism for the reaction of the OH radicals with the isoalloxazine moiety has been discussed. The rate constant for the reaction of isoalloxazine moiety of FAD with OH radicals was determined to be 4.7×10^{9} dm^{3} mol^{-1} s^{-1} by competition kinetic studies using glucose as the reference solute. From the kinetic consideration and G-values it is evident that ca. 50% of the OH radicals attack at the AMP (adenosine monophosphate) portion of FAD apart from isoalloxazine moiety. The attack at the AMP portion of FAD is supported by the observed phosphate release in the irradiated FAD solution. Effect of Fe^{3+} ion on the radiolysis of FAD and FMN was also studied.

A large number of dehydrogenase enzymes which are very important in biological functions, e.g., electron transport in mitochondria, biosynthesis of pyrimidine bases, etc. contain flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) as their prosthetic groups. When exposed to ionizing radiation these enzymes lose their activity. But actual mechanism of inactivation of these flavoenzymes is yet to be known. To have an insight on the mechanism of radiation inactivation it is therefore necessary to study the chemical changes produced by radiation on free FMN and FAD molecules.

Winstead and Moss1) have studied the inactivation of FAD by γ-irradiation in dilute aqueous solution both in presence and absence of air. From the spectral changes owing to varying doses absorbed they have suggested that the inactivation is due to attack by the water-borne free radicals derived from water radiolysis ($H_2O \rightsquigarrow$ H, OH, e^-_{aq})2) at the isoalloxazine moiety of FAD.

In this paper we present our investigation on the role of the OH radicals in the decomposition of FAD and FMN in terms of G-values, the rate constant for the reaction of the OH radicals with isoalloxazine moiety of FAD, the possible sites of attack of OH radicals and the effect of iron(III) ion on the radiolysis of FAD and FMN in dilute aqueous solution.

Experimental

Materials. FAD and FMN were purchased from Sigma Chemical Company. Glucose, ferric ammonium sulfate, and other reagents that were used in the investigation were of 'AnalaR' grade. Triply distilled water was used to prepare the solutions. A phosphate buffer (2×10^{-2} mol dm^{-3}) was used for maintaining the pH at 6.5 of FAD and FMN solutions. Acetonitrile was of HPLC grade (Aldrich). Argon and N_2O gases of high purity were used for flushing the solutions. A ^{60}Co γ-source (dose rate=12 or 3.3 Gy min^{-1}) was used for irradiating the solutions. The dose rate was measured by Fricke dosimetry.

Analysis. The loss of flavin chromophore in FAD and FMN on γ-irradiation was estimated spectrophotometrically by measuring the absorbance of their solutions at 450 nm. The spectra of FAD and FMN were measured on a recording spectrophotometer. In the case of absorbance measurements of FAD and FMN in argon or N_2O saturated solutions a stoppered quartz spectrophotometer cell was used. The loss of FMN was also determined by measuring the loss of fluorescence intensity at 530 nm using an excitation wavelength of 450 nm.

The amount of FMN and FAD in irradiated samples was ascertained by separating them from their radiolysed products by HPLC. The HPLC separation was done by using Waters Liquid Chromatograph with an ODS-C_{18} column. A mobile phase of 1×10^{-2} mol dm^{-3} disodium hydrogenphosphate (adjusted to pH 5.5 with dilute H_3PO_4)-acetonitrile (100 : 12) was filtered, degassed, and used at a flow rate of 1.0 mL min^{-1}. The column eluents were monitored by the UV absorption at 450 nm and 210 nm using a variable wavelength detector.

Inorganic phosphate release from FAD after irradiation was measured by the method of Lowry and Lopez.3)

Iron(II) ion concentration was measured spectrophotometrically by means of 1,10-phenanthroline reagent.4)

Results

Figure 1a—c illustrates the effect of γ-irradiation on the spectral properties of 2.5×10^{-5} mol dm^{-3} FAD (pH 6.5) in aerated, N_2O saturated and argon saturated solutions. The spectrum after aeration of the irradiated solution has been shown in each case. It is evident that under argon saturated conditions, the absorbance decreased due to irradiation are partially restored by aeration of the irradiated samples. This suggests that some products formed radiolytically are partially oxidized to regenerate the parent molecule. Similar observations have also been obtained in the case of FMN.

Figure 1d shows that the loss of chromophore in FAD is not linear with the increase of the absorbed dose. The initial G-values for flavin chromophore loss in FAD have been determined from the initial slope of the

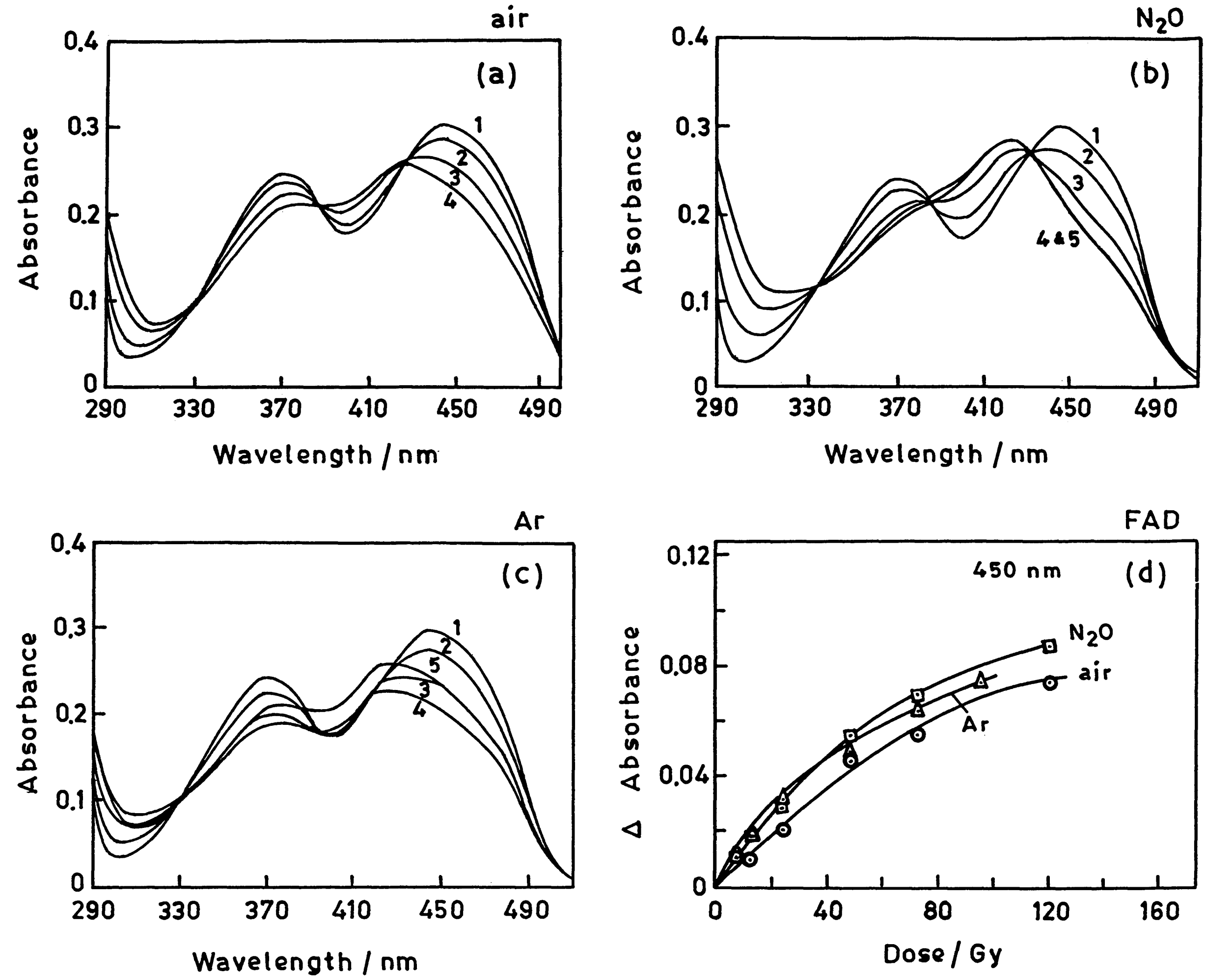


Fig. 1. Absorption spectra of FAD (2.5×10^{-5} mol dm^{-3}) in 2×10^{-2} mol dm^{-3} phosphate buffer irradiated under aerated (a), N$_2$O-saturated (b), and argon saturated (c), conditions. Absorbed dose: 1: 0.0 Gy; 2: 24 Gy; 3: 72 Gy; 4: 120 Gy 5: spectra obtained after aeration of the FAD solution irradiated with a dose of 120 Gy. (d) Changes of A_{450} for FAD solution with absorbed dose under aerated (⊙), N$_2$O-saturated (⊡), and argon saturated (◬) solutions.

curves. The flavin chromophore loss in FMN was also determined in the same way. The results are presented in Table 1.

The fluorescence spectra of unirradiated and irradiated FMN solutions are shown in Fig. 2. This figure shows that on irradiation the fluorescence intensity decreased but there was no change in spectral shape of irradiated samples from the unirradiated one. The loss in fluorescence intensity was found to be linear with absorbed doses. Hence G(-FMN) values in aerated and N$_2$O saturated conditions were determined from the slope of the linear plot of loss of fluorescence intensity versus absorbed dose. The G(-FMN) values determined from such fluorescence measurements are in good agreement with those determined from HPLC analysis (Table 1) and from absorbance measurements. Therefore it would appear that the spectrophotometric determination of G-values from the disappearance of FMN absorbance is relatively free from significant error. In the case of FAD, although the spectral shape of the fluorescence remained same, there was increase in the fluorescence intensity at 530 nm after gamma irradiation of low doses. Hence the loss of flavin moiety in FAD on irradiation could not be determined fluorometrically. Further, the loss of FAD measured by HPLC differed markedly from that by loss of absorbances. Hence the decrease of absorbance at 450 nm gives only the extent

Table 1. G-Values for Flavin Loss under Different Conditions, [FAD]=[FMN]=2.5×10^{-5} mol dm^{-3}, pH=6.5

G-Values	Solution saturated with		
	Air	Argon	Nitrous oxide
G(-chromophore)$_{\mathrm{FMN}}$[a]	1.23	2.1(1.20)[b]	
G(-chromophore)$_{\mathrm{FAD}}$[a]	0.68	1.38(0.70)[b]	1.30
G(-FMN)	1.16(1.10)[c]	—	2.8(2.66)[c]
G(-FAD)	1.30	—	2.50

a) Determined spectrophotometrically. b) Measured after aeration of the radiolysed solution. c) Determined fluorometrically.

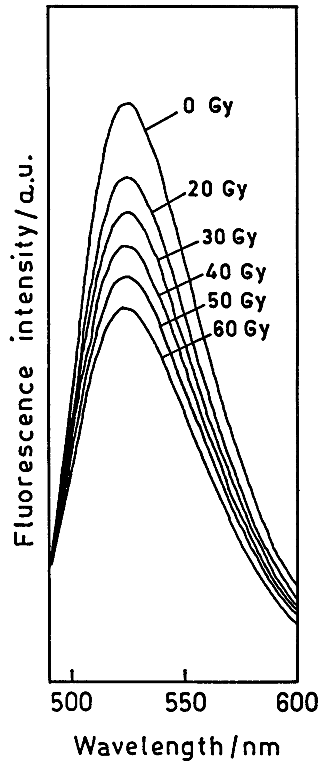


Fig. 2. Changes in the fluorescence emission of FMN (2.5×10^{-5} mol dm^{-3}) solution after γ-irradiation under aerated conditions. Excitation wavelength= 450 nm.

of modification of flavin chromophore in FAD. That is why *G*-values determined spectrophotometrically and by HPLC are expressed as *G*(-chromophore) in the former case and by *G*(-FAD) or *G*(-FMN) in the latter case.

Since some products generated on irradiation of FAD and FMN under argon are found to be reverted back by aerating the irradiated solution, the *G*-values in these cases could not be measured by HPLC.

The rate constant for the reaction of isoalloxazine moiety in FAD with the OH radicals has been determined by the competition kinetic technique using glucose as the reference solute. When irradiation was carried out in N_2O-saturated medium, e_{aq}^- reacts exclusively with N_2O to give a stoichiometric equivalent of OH radicals.

$$e_{aq}^- + N_2O \longrightarrow N_2 + OH + OH^-. \qquad (1)$$

Therefore, when an aqueous solution of FAD and glucose is irradiated in N_2O saturated conditions, there would be a competition of FAD and glucose (GLH) for the OH radicals. The yields of chromophore loss of FAD should then follow the relationship

$$\frac{G(\text{-chromophore})_0}{G(\text{-chromophore})} = 1 + \frac{k_1}{k_2}\frac{[\text{GLH}]}{[\text{FAD}]}, \qquad (2)$$

where *G*(-chromophore)$_0$ and *G*(-chromophore) are the yields of chromophore loss of FAD in the absence and

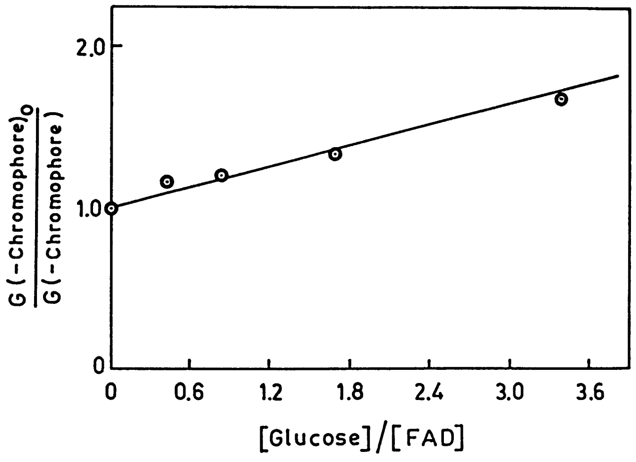


Fig. 3. Kinetic plot for the competition reactions of OH radicals between FAD and glucose. [FAD]= 2.5×10^{-5} mol dm^{-3}.

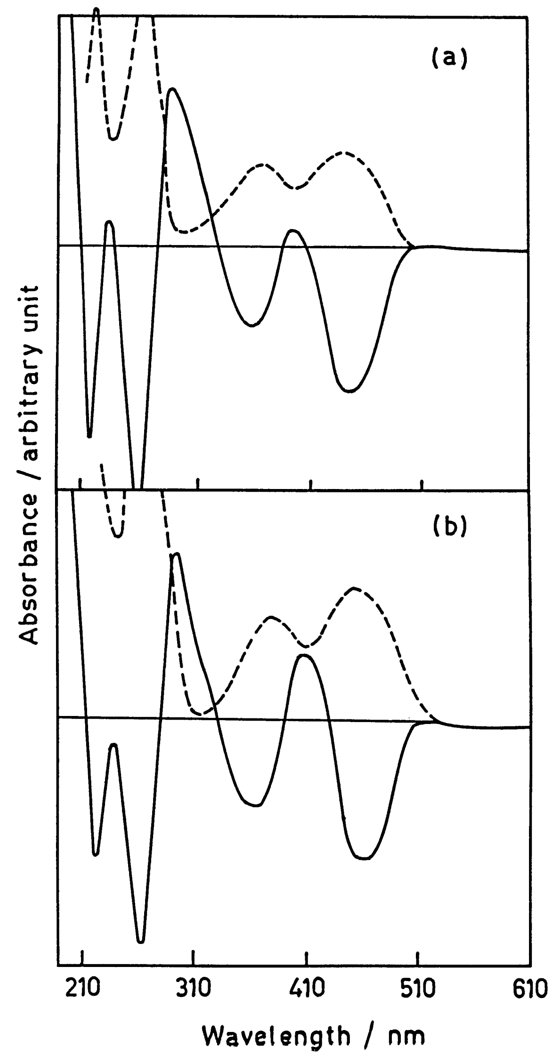


Fig. 4. Difference spectra of the products formed in FAD, (a), and FMN, (b) solutions irradiated with a dose of 120 Gy under N_2O-saturated solutions. Dashed curves are the respective absorption spectra.

presence of glucose, respectively; [GLH] and [FAD] are the molar concentrations of glucose and FAD, k_1 and k_2 being the rate constants for the reactions of OH radicals with glucose and the isoalloxazine moiety in FAD respectively. If G(-chromophore)$_0$/G(-chromophore) is plotted against [GLH]/[FAD], a straight line with a slope of k_1/k_2 is expected. The observed results are plotted in Fig. 3. From the slope of straight line the value of k_1/k_2 could be determined. Taking the value of k_1 as 1×10^9 $\mathrm{dm^3\,mol^{-1}\,s^{-1}}$,[5] the value of k_2, the rate constant for the reaction of isoalloxazine moiety in FAD with the OH radicals, has been estimated to be 4.7×10^9 $\mathrm{dm^3\,mol^{-1}\,s^{-1}}$.

The difference spectra of irradiated FAD and FMN in N_2O saturated medium are shown in Fig. 4a,b. The figure shows that in both cases there are three peaks at nearly identical wavelengths 408, 296, and 240 nm. The similarity of the product peaks obtained under the similar experimental conditions indicates that the same types of products have been formed in both cases.

As some of the flavoenzymes contain iron(III) the role of this metal ion in modification of radiosensitivity of flavins was also studied. Because of the instability of iron(III) ions in aqueous solution at pH 6.5 the study was to be carried out at pH ca. 2. The role of Fe^{3+} ions at pH ca. 2 on isoalloxazine moiety of FAD and FMN could be extrapolated to pH 6.5, since the pK value for $Fl+H^+=FlH^+$ is nearly zero[6] (where Fl represents flavin moiety). Therefore, flavin would remain in a neutral state at both pH 6.5 and ca. 2. Thus, when FAD is irradiated in the presence of iron(III) ion in argon saturated medium at pH ca. 2 the loss of flavin chromophore in FAD has been found to be the same as that obtained in absence of iron(III) ion. But the reduction yield of iron(III) ion $G(Fe^{2+})$ was found to increase with the increase in its concentration and reached a plateau value of ca. 4.8 at higher $[Fe^{3+}]$ as shown in Fig. 5. The reduction yield of iron(III) ion in the presence of FMN was found to be identical with that observed in the presence of FAD under similar experimental conditions.

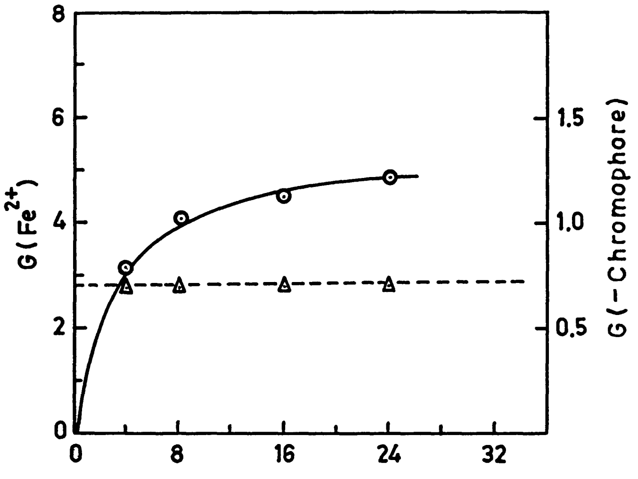


Fig. 5. Dependence of $G(Fe^{2+})$, (⊙) and G(-chromophore) in FAD, (△) on concentration of Fe^{3+} ion in the γ-radiolysis of FAD in the presence of Fe^{3+} ions at pH ca. 2 under argon saturated conditions, $[FAD]=2.5\times10^{-5}$ $\mathrm{mol\,dm^{-3}}$.

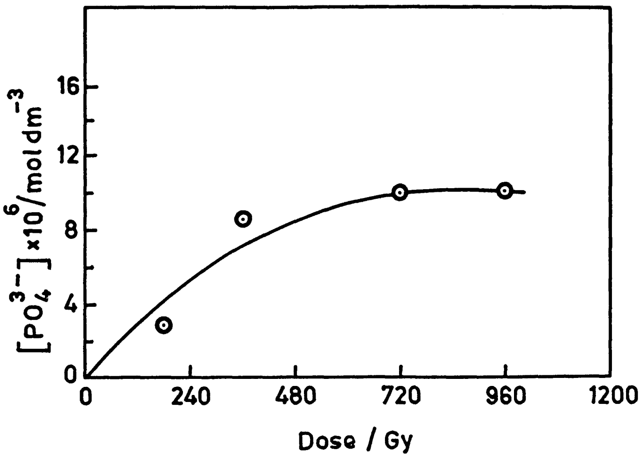


Fig. 6. Extent of phosphate release from FAD on γ-radiolysis under N_2O-saturated conditions, $[FAD]=2.5\times10^{-4}$ $\mathrm{mol\,dm^{-3}}$.

In addition to the flavin moiety the FAD molecule also contains a phosphate–sugar linkage. It is well-known that when OH radicals react with the sugar moiety of nucleotides, inorganic phosphates are released.[7] To ascertain whether OH radicals react with FAD at sites other than the isoalloxazine ring, the amount of inorganic phosphate released in irradiated FAD was determined. Figure 6 shows the extent of phosphate release in γ-irradiated FAD in N_2O saturated medium against the absorbed dose. The initial G-value for the phosphate release was estimated to be ca. 0.23. But no detectable amount of phosphate release was observed on γ-irradiation of FMN under the similar conditions.

Discussion

In the aerated medium e_{aq}^- and H radicals are scavenged by oxygen forming the superoxide anion (O_2^-) and hydroperoxyl (HO_2) radicals as given in the Eqs. 3 and 4.

$$e_{aq}^- + O_2 \longrightarrow O_2^- \qquad (3)$$

$$H + O_2 \longrightarrow HO_2^{\cdot} \qquad (4)$$

Since the redox potential of O_2/O_2^- ($E_0=-0.33$ V)[8] is lower than those of Fl/Fl^- ($E_0=-0.3$ V),[9] the superoxide anions could reduce flavins to dihydroflavins. But these dihydroflavins would be oxidized readily by molecular oxygen by a complex mechanism[10] to give back flavins. Hence there would be no net modification of flavins due to reaction with O_2^- in aerated medium.

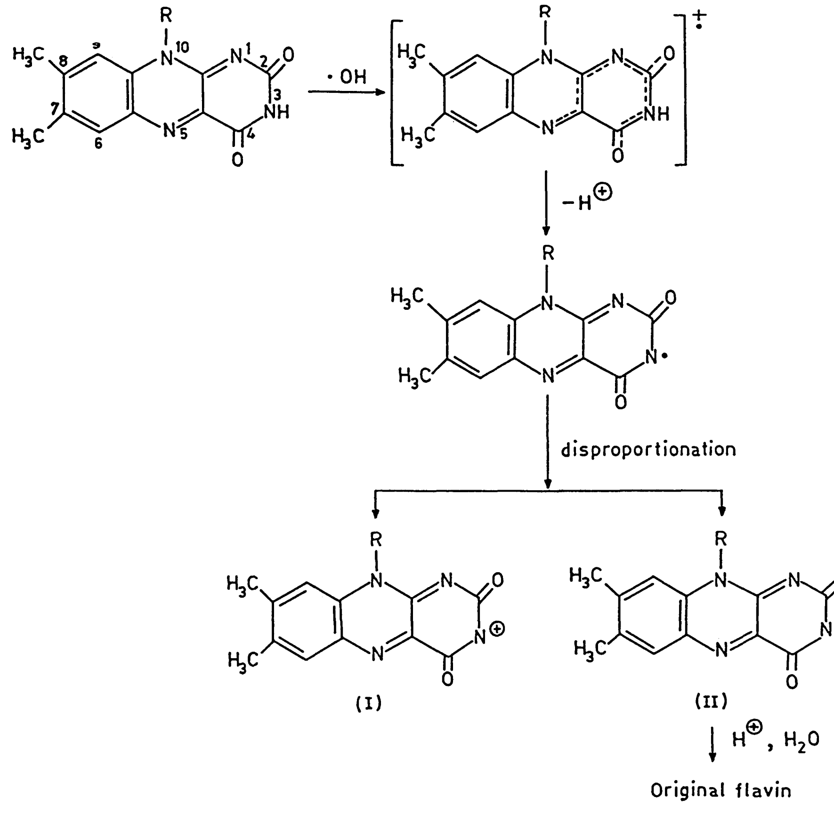
In N_2O saturated medium G_{OH} is twice than that in aerated medium. From Table 1, it is seen that the chromophore loss in FAD and FMN in N_2O saturated medium are nearly twice that observed in aerated medium. This result definitely shows that OH radicals have important contribution to the modification of FAD and FMN during the irradiation by γ-rays. The flavin chromophore loss of FAD and FMN under the deaerated conditions was found to be larger than that observed under the aerated conditions (Table 1). This may be attributed to the reactions of FAD with e_{aq}^- and H radicals. Ahmad et al.[11] have shown that FAD and FMN are reduced by e_{aq}^- to form dihydroflavins, FlH_2.

$$\mathrm{Fl} + e_{aq}^- + H^+ \longrightarrow \mathrm{FlH} \qquad (5)$$

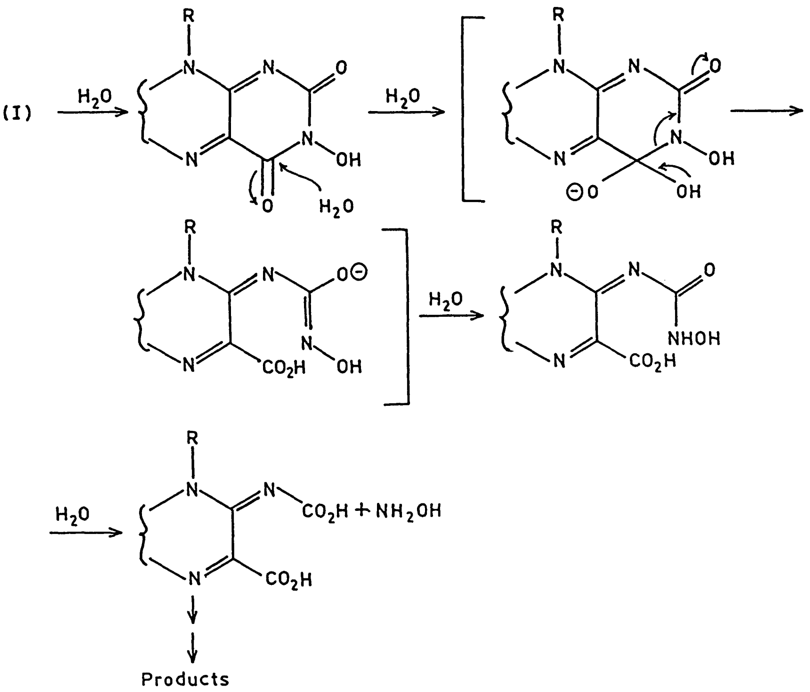
$$2\,\mathrm{FlH} \xrightarrow{\text{disproportionation}} \mathrm{Fl} + \mathrm{FlH_2} \qquad (6)$$

As stated earlier these dihydroflavins are oxidized by molecular oxygen to flavins. This explains the partial recovery of absorbances of irradiated FAD and FMN by aeration as shown in Fig. 1c. But in the case of dinitrogen monoxide saturated condition the absorption spectra of the irradiated sample remained unchanged (Fig. 1b) on aeration. Since the major damaging species in N_2O saturated medium is OH radicals, it could be inferred that OH radicals cause a permanent damage to FAD and FMN. This permanent change in absorption caused by OH radicals might be due to modification of the isoalloxazine moiety of flavins. The absorption behavior of the products formed from such reactions could be studied following the difference spectra of the products formed in the reactions. Figure 4 shows that there are three negative peaks in the difference spectra of the products with minima at those wavelengths (260, 370, and 450 nm) which correspond to the maxima in the absorption spectrum of unirradiated FAD and FMN. These negative peaks indicate the removal of ground-state absorption of FAD and FMN on radiolysis. The absorption peaks of the products have shifted to shorter wavelengths than those of starting flavins. This spectral shift may imply that OH radical reaction causes the loss of π-conjugation of isoalloxazine moiety of flavins.

From Table 1 it is evident that under aerated or N_2O saturated conditions the decomposition yield of flavins, viz. FMN is equal to half the value of G_{OH}. To account for this G-value, a disproportionation reaction of the transient intermediate formed by the reaction of OH radicals with the flavin should take place as was suggested previously.[12] Heelis et al.[13] have suggested that possible oxidation sites at the flavin moiety are the C-8 methyl group and the extended π-conjugated system. They have shown that $SO_4^{-\cdot}$ radical causes oxidation predominantly by abstracting electron from the π-conjugated system followed by deprotonation from N-3 atom. Kishore et al.[14] have shown by pulse radiolysis studies that OH radical causes oxidation only at π-conjugated system in flavin moiety of lumichrome. To explain the chromophore loss in FAD and FMN an analogous mechanism may be shown as Schemes 1 and



Scheme 1.



Scheme 2.

2.

But the above mechanism cannot account for the observed G(-chromophore) in FAD. Table 1 shows that under N_2O saturated conditions the G(-chromophore) for FAD is only 1.3 which is much less than the value expected from the reaction mechanism. The possible reason for this discrepancy is that apart from the isoalloxazine moiety a significant portion of the total OH radicals attacks at the AMP (adenosine monophosphate) portion of FAD. The rate constant for the reaction of OH radicals with adenosine (ca. 3.7×10^9 $dm^3\,mol^{-1}\,s^{-1}$) is comparable to that with flavin moiety of FAD and calculation shows that ca. 50% of the total OH radicals would react with the AMP moiety of FAD molecule. This is supported from the fact that in both aerated and N_2O saturated media the G(-chromophore) values in FAD are nearly half of that in FMN under identical conditions. However, when the loss of FAD was measured after separating the products by HPLC, G(-FAD) was found to be ca. 2.5, which is much higher than the value obtained from the spectrophotometric measurements. This increased degradation of FAD is due to the modification of AMP moiety in FAD. The attack of OH radicals at the AMP portion is further confirmed from the phosphate release on γ-irradiation of FAD in N_2O saturated medium, because the attack of OH radical on the AMP results in phosphate release from the molecule.

The role of Fe^{3+} in the radiolysis of FAD and FMN has also been studied. In both cases the reduction of Fe^{3+} has been observed. At pH ca. 2 under deaerated conditions, the $G(Fe^{2+})$ value in absence of FAD and FMN was 3.0 which is almost equal to the radiation chemical yield of the H atoms.[2] But in the presence of FAD or FMN the $G(Fe^{2+})$ value was found to be 4.8. This increase of the $G(Fe^{2+})$ value might be due to the reaction of Fe^{3+} with the transient intermediate formed by the reaction of OH radicals with flavins or by other reducing products formed in the reaction. It is interesting to note that the chromophore loss in FAD and FMN were not influenced by the presence of Fe^{3+} during irradiation. Hence the reduction of Fe^{3+} by the transient radical is unlikely. The only possible pathway to account for the formation of reduced iron is the reduction of Fe^{3+} by the product formed in the solvolysis of (I) (Schemes 1 and 2). Hydroxylamine or the hydroxamic acid derivative (III) could reduce two equivalents of Fe^{3+}. Thus according to the aforesaid discussion $G(Fe^{2+})$ is expected to be $G_H+2\times G$(-chromophore)$=3.0+2\times1.2=5.4$ for FMN and $G_H+2\times G$(-chromophore)$=3.0+2\times0.7=4.4$ for FAD. If a small loss of Fe^{2+} ion due to aereal oxidation during measurements is considered, then the observed results are found to be in conformity with that expected in the case of FMN. But for the case of FAD, the observed $G(Fe^{2+})$ is ca. 4.8 (Fig. 5) which is slightly higher than the expected value. It is important to mention here that the attack of OH radicals towards AMP would produce radicals which are easily reduced and oxidized in nature.[15] Thus reducing radicals might react with Fe^{3+} ions giving Fe^{2+}. As a result, the yield of Fe^{2+} could be increased without affecting G(-chromophore) of FAD molecule.

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