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Photobiology

STUDY OF RADIATION DAMAGE AND PROTECTION OF SOME VITAMINS IN AQUEOUS MEDIA*

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INTRODUCTION

Recently a lot of interest has been evinced in the radiation sterilization of biochemicals. This process has several advantages over the traditional autoclaving process (Iya, 1973). But radiation, while it destroys micro-organisms, also interacts directly or indirectly with the compound being sterilized. This may result in destruction of the compound and simultaneous formation of various products. In dilute aqueous solutions, ionizing radiations such as ϒ-rays, interact with water producing the highly reactive short-lived species: H atoms, OH radicals and hydrated electrons (e^-_{aq}). These can react with the solute giving new products which may or may not be toxic. It has been found that aqueous solutions of many biochemicals such as vitamins, antibiotics and other drugs undergo extensive degradation upon ϒ-irradiation (IAEA, 1967). In those pharmaceutical preparations in which water is one of the constituents, radiation sterilization would be detrimental to the potency of the preparation, unless additives are incorporated which can scavenge these reactive species, to give secondary products that do not react with the active component of the preparation.

In order to be able to protect the biochemical from radiolytic degradation, the additives should have a higher or equivalent reactivity with these free radicals than the biochemical itself. Further, from the practical viewpoint the additive as well as its radiolytic products should not be toxic and they should not also reduce the microbiological activity of the vitamin. Sulphur containing compounds like Glutathione have been used as protective agents in the past (Barron & Dickman, 1949). Thus at a dose of 5 Krads the suppression in the enzyme activity of succinoxidase was 95%, whereas in presence of glutathione the suppression was only 23%. In the case of vitamin B_{12}, Blackburn *et al.* (1972) have used formate ion (0.1M) and oxygen to protect the vitamin. In such a system OH radicals react with $HCO\bar{O}$ giving CO_2^- radical. The resulting reducing species CO^-_2 as also e^-_{aq} react with vitamin B_{12} to give vitamin B_{12r}, which can be oxidized back to vitamin B_{12} by oxygen.

We have tried glucose for scavenging OH radicals and oxygen or nitrous oxide for e^-_{aq}. In pulse radiolysis experiments it has been observed (Moorthy & Hayon, unpublished) that only a small fraction (<0.1) of glucose radicals (concentration$\sim 10^{-6}$ molar) react with the B-group vitamins (concentration$\sim 10^{-3}$ molar), thus making it a suitable

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choice as an additive. The radiolysis products of glucose in aqueous solutions have also been found to be non-toxic (Schubert, 1974).

Materials and Methods

All chemicals used were either analar or 'A' grade. Solutions (10^{-4} molar) in vitamins were prepared in phosphate buffer using triply distilled water. The *p*H in all cases, except pantothenic acid (used as the hemi-calcium salt) was 6.8. In the case of pantothenic acid it was 6.0 as this compound is unstable at higher *p*H. The solutions were made 0.1M with respect to glucose wherever necessary and flushed with oxygen, nitrous oxide or nitrogen as required. Solutions were prepared just prior to use and were protected from room light. The uv-spectra were taken before and after γ-irradiation on Beckman DU spectrophotometer. Irradiations were performed in a ^{60}Co γ-source at a dose rate of 0.2 Mrad/hr.

Results and Discussion

Figs. 1 and 2 show spectra of various vitamin solutions γ-irradiated in presence of air to a dose of 50 Krads. Comparison of these spectra with those of the unirradiated solutions reveals extensive degradation of the vitamins. These spectral changes are in qualitative accord with those reported in the literature.

Early work on the radiolysis of vitamins does not give much information regarding the products formed or the mechanism of destruction. Sjoestedt & Ericson 1962 have studied the effect of γ-radiation on a number of water-soluble vitamins, viz. thiamine, riboflavin, nicotinamide, biotin and folic acid. But they have not discussed the mechanism of degradation of these vitamins in aqueous solutions.

The bimolecular rate constants for the reaction of e^-_{aq} and OH with the various vitamins, N_2O, O_2 and glucose are given in Table I. From Table I it can be seen that in N_2O or oxygen saturated aqueous solutions of vitamins of concentrations $\leq 10^{-3}$ molar, the hydrated electrons are selectively scavenged by these additives according to

$$e^-_{aq} + N_2O \xrightarrow{H_2O} N_2 + OH + OH^- \quad \ldots (1)$$

$$e^-_{aq} + O_2 \longrightarrow O_2^- \quad \ldots (2)$$

(Saturation solubility of N_2O in water is~25 m molar and that of oxygen ~ 1 m molar.)

Table I

Bimolecular rate constants for the reaction of e^-_{aq} and OH

Compound	e^-_{aq} rate constant		OH rate constant		Reference
	*p*H	K(M^{-1} Sec^{-1})	*p*H	K(M^{-1} Sec^{-1})	
Thiamine	6.1	3.4×10^{10}	6.6	3×10^{9}	(8)
Pyridoxin	6.8	2.2×10^{10}	7.2	6.3×10^{8}	(9)
Folic acid	6.0	2.2×10^{10}	6.0	3×10^{10}	(10)
Nicotinamide	7.5	2.4×10^{10}	—	—	(11)
Riboflavin	7.0	2.3×10^{10}	—	—	(12)
Pantothenic acid	6.6	1.2×10^{8}	6.6	4.5×10^{9}	(13)
Glucose	—	1×10^{6}	7.0	1×10^{9}	(14)
N_2O	7.0	5.6×10^{9}	—	—	(14)
O_2	7.0	1.88×10^{10}	—	—	(14)

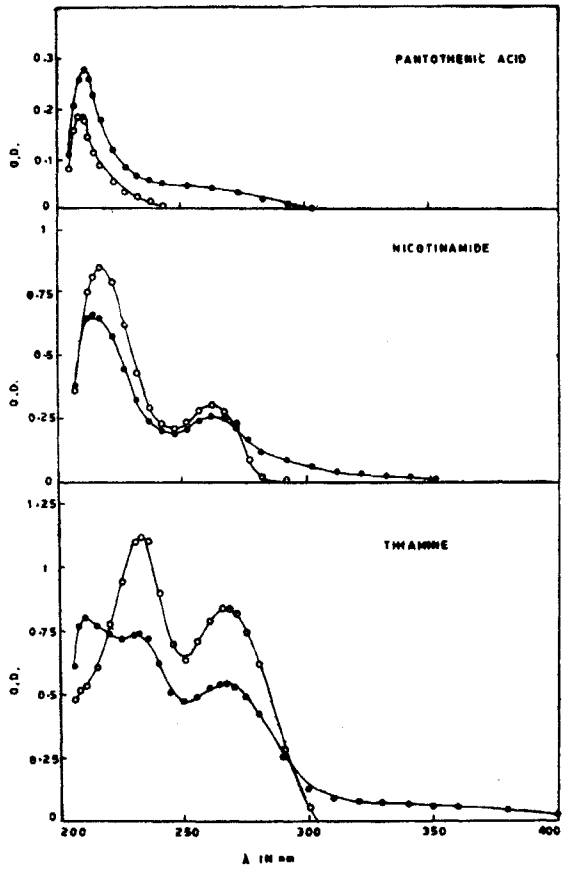


FIG. 1 Absorption spectra of 10^{-4}M vitamin solutions ($pH = 6.8$).
0—unirradiated, ●—γ-irradiated in air.

Glucose in 0.1 M concentration is able to selectively scavenge the OH radicals. The sugar radicals so formed have a higher redox potential (e.g. E_o of ribose radicals is +0.06 volts (Rao & Hayon, 1974) as compared to those of the vitamins (see Table II)

TABLE II

Redox potentials of various vitamins (calculated from $E_{\frac{1}{2}}$ data of Ref. 16)

Vitamin	E'_o (volts)
Thiamine	— 0.97
Pyridoxine	— 1.6
Folic acid	— 0.57
Nicotinamide	— 1.2
Reboflavin	— 0.21
Pantothenic acid	— 1.3

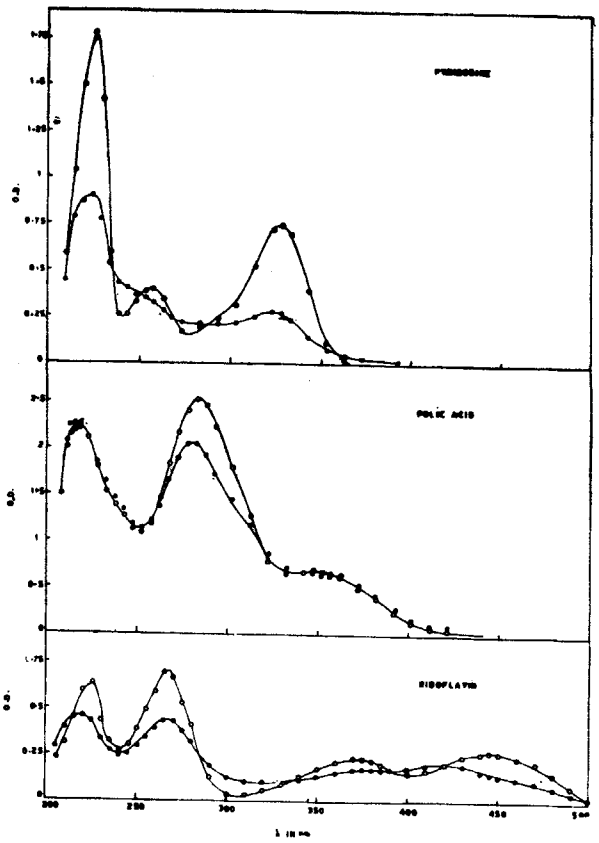
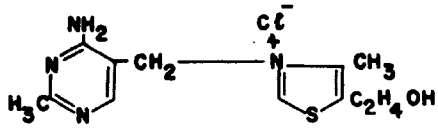


FIG. 2. Absorption spectra of 10^{-4}M vitamin solutions (pH=6.8).
0—unirradiated, ●—γ-irradiated in air.

and hence are not expected to react with the latter. This was confirmed in pulse radiolysis experiments. The same is true of O_2^- formed by reaction (2) $E_o = +0.15$ volts (Rao & Hayon, 1973). It is thus evident that the use of N_2O and glucose or O_2 and glucose should provide protection to the vitamins from radiolytic degradation in aqueous solutions. The results obtained amply confirm these predictions.



THIAMINE

Fig. 3a shows the uv spectrum of unirradiated 10^{-4} M thiamine at pH 6.8, with its characteristic absorption maxima at 232 and 267 nm.

Fig. 3b shows the uv spectrum of 10^{-4} M thiamine solution, γ -irradiated after saturation with N_2O. Under this condition e_{aq}^- reacts exclusively with N_2O to give a

stoichiometric equivalent of OH radicals and the products expected are those formed by the reaction of these radicals with thiamine. This leads to destruction of thiamine as is evident from the spectra. The products may have an absorption maximum below 210 nm and exhibit a tailing absorption into the visible region.

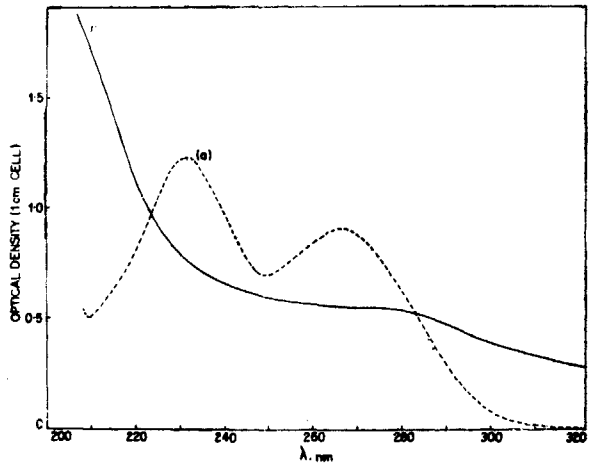
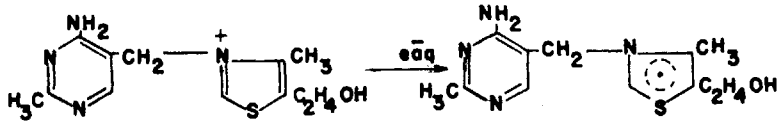


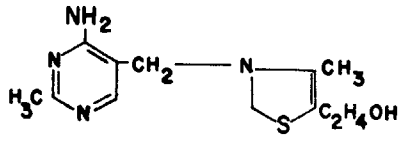
FIG. 3. Absorption spectra of 10^{-4}M thiamine solution (pH=6.8).
(a)——unirradiated, (b).....γ-irradiated in presence of N_2O.

Fig. 4a shows the uv spectrum of 10^{-4} M thiamine, 0.1M glucose (pH=6.8) irradiated under N_2. Under these conditions, the hydrated electrons react with thiamine to produce ultimately dihydrothiamine. The hydroxyl radicals react with glucose to give as final products gluconic and glucorunic acids, arabinose, erythrose, glyoxal and dihydroxyacetone. Dihydroxyacetone has a characteristic absorption at 265 nm (Phillips, 1963). This is shown in Fig. 4b which is the spectrum of 0.1 M glucose solution pH=6.8, γ-irradiated under nitrogen. On point by point substraction of curve 4b from 4a, the curve 4c is obtained. This is very similar to the spectrum of dihydrothiamine with maxima at 235 and 275 nm.

Ueno and Fukuda (1962) have studied the γ-radiolysis of thiamine. They observed more decomposition in presence of oxygen, than in presence of nitrogen. Addition of glutathione reduced the decomposition considerably. In the pulse radiolysis study of thiamine and related compounds in aqueous solutions, it has been inferred (Moorthy & Hayon, *in press*) that e^-_{aq} reduces the thiazolium ring of thiamine to give the electron adduct radical.



This radical decays by second-order kinetics, presumably disproportionation, to give dihydrothiamine (Maier & Metzler, 1957).



DIHYDROTHIAMINE

Our observation of dihydrothiamine as a product of γ-irradiation of the system 10^{-4} M thiamine, 0.1, M glucose, N_2, is in agreement with this conclusion. The spectrum of the transient produced by OH radical reaction with thiamine has also been observed but not identified. The OH radicals are expected to add primarily to the pyrimidine ring and to the thiazolium ring (possibly at C_2 position).

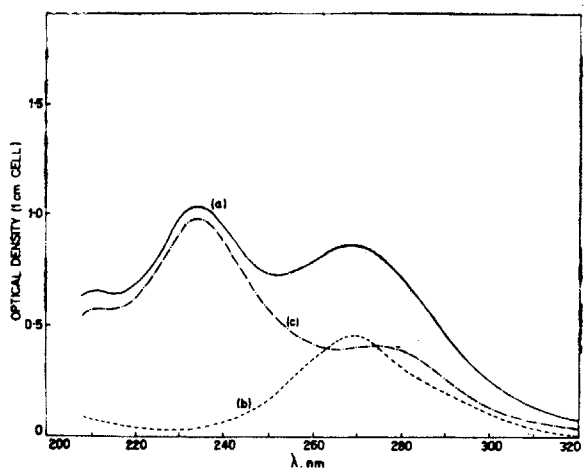


FIG. 4. Absorption spectra of γ—irradiated aqueous solutions (pH=6.8).
(a)—— 10^{-4}M thiamine+0·1M glucose, N_2 atmosphere,
(b)..... 0.1M glucose, N_2 atmosphere,
(c).-.-.- (a)—(b).

In addition, H atom abstraction by OH from the side chain may occur. The radical produced decays by second-order kinetics with $2K=1.6 \times 10^8$ M^{-1} Sec at pH=6.7. Further work is in progress to identify the products formed.

Figs. 5a and 5b show the spectra of 0.1 M glucose, and 10 M thiamine, 0.1 M glucose solution (pH=6.8), irradiated-under N_2O atmosphere. The former spectrum is that of radiation products from glucose, mainly dihydroxyacetone. The higher (almost double) intensity of the 265 nm peak as compared to the spectrum shown in Fig. 4a is as expected. On subtraction of 5a from 5b, the spectrum 5c was obtained; this is seen to be identical with the spectrum of the unirradiated 10^{-4} molar thiamine solution. It is

thus evident that in presence of N_2O and glucose, thiamine does not undergo radiolytic degradation. Instead of N_2O, one can employ oxygen for scavenging e^-_{aq}, provided that O_2^- radicals formed in reaction (3)

$$O_2 + e^-_{aq} \longrightarrow O_2^- \qquad \ldots (3)$$

do not reduce thiamine. From the reduction potential of O_2^-($E_o = +0.15$ volt) this is expected to be the case. The spectrum of 10^{-4} molar thiamine, 0.1 molar glucose solution (pH=6.8), irradiated under oxygen atmosphere is found to be identical with that of unirradiated 10^{-4} M thiamine solution. In presence of oxygen the product from glucose which absorbs at 265 nm is not formed (Phillips 1963). The spectrum of the 0.1 M glucose solution irradiated under O_2 atmosphere shows hardly any absorption over the region 210 to 350 nm. The radiolysis of oxygen-saturated aqueous solution of glucose can be represented by equations 4 to 7.

$$RCH_2OH + OH \rightarrow RCHOH + H_2O \qquad \ldots.(4)$$

$$RCHOH + O_2 \rightarrow \underset{\displaystyle O_2}{\underset{|}{RCHOH}} \rightarrow RCHO + HO_2 \qquad \ldots.(5)$$

$$RCHO + HO_2 \rightarrow RCOOH + \tfrac{1}{2}H_2O_2 \qquad \ldots.(6)$$

$$O_2 + e^-_{aq}(H) \rightarrow O_2^- (HO_2) \qquad \ldots.(7)$$

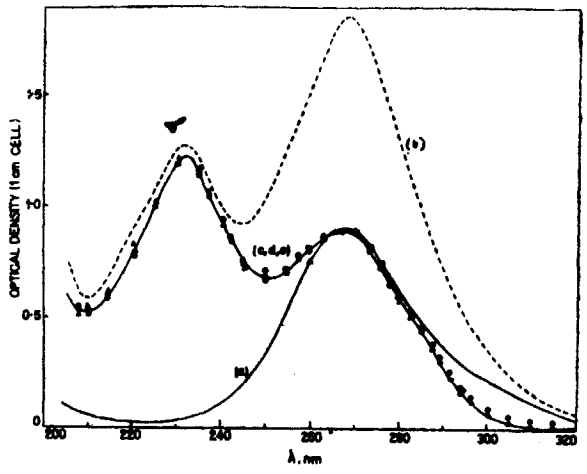
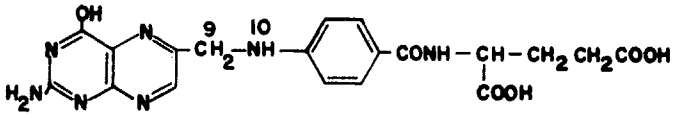


FIG 5 Absorption spectra of γ-irradiated aqueous solutions (pH=6.8).
(a)———0.1M glucose, N_2O atmosphere,
(b).....10^{-4} M thiamine, 0.1M glucose, N_2O atmosphere, (c) △—(b)–(a).,
(d) ●—10^{-4} M thiamine, 0.1M glucose, oxygen atmosphere;
(e) ○—unirradiated 10^{-4}M thiamine, 0.1M glucose (pH=6.8).

From this scheme the hydrogen peroxide yield $G(H_2O_2)$ (no. of molecules formed per 100 ev of energy absorbed) is expected to be 3.6 from the known yields of e^-_{aq}, H and OH.

We have measured the H_2O_2 yields in 0.1M glucose alone and with 10^{-4} M thiamine sent, both irradiated under O_2. In both cases the value obtained was $G(H_2O_2)=3.3$. These results confirm the conclusion based on the uv spectra that O_2^- (or HO_2) does not destroy thiamine, but reacts with glucose radicals instead. Thus radiation protection of thiamine is also possible in the oxygenated glucose system.



FOLIC ACID

Naaken and Phil (1966) and Galatzeanu (1966) have studied the radiolysis of folic acid. It has been suggested that OH radicals abstract hydrogen atom from carbon atom C_9. The resulting radical may interact with oxygen and the peroxide radical formed may add to the benzene ring in the ortho-amino position. In the subsequent dismutation reaction, 3-hydroxy para-aminobenzoyl glutamic acid and 4-hydroxy 6-formyl pteridine may be formed. Pulse radiolysis of deaerated solutions of folic acid (Moorthy & Hayon, *in press*) containing t-butanol as OH scavenger, showed the formation of a transient species identified as the protonated electron adduct of folic acid.

Fig. 6 depicts the spectra of unirradiated folic acid (pH = 6.8) and of folic acid γ -irradiated to 0.15 Mrad, under N_2O to show the OH radical reaction. On the other hand, when irradiated in presence of glucose and nitrogen only the hydrated electron reaction with folic acid takes place. It can be seen from the spectra that there is degradation in both the cases. We have not identified the products yet.

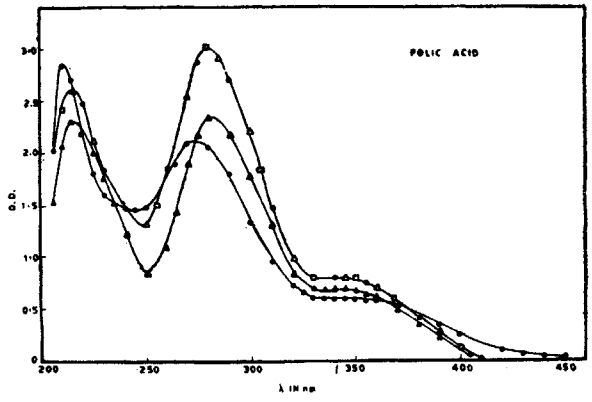


FIG. 6. Absorption spectra of 10^{-4}M folic acid solution (pH = 6.8). o—unirradiated; ●—γ-irradiated in presence of N_2O, △—γ-irradiated in presence of 0.1M glucose 0_2; □—γ-irradiated in presence of 0.1M glucose and N_2O (spectrum corrected for the absorption of irradiated glucose solution); ▲—γ-irradiated in presence of 0.1M glucose and N_2 (spectrum corrected for the absorption of irradiated glucose solution).

In the same figure it can be seen that there is no degradation of the vitamin when it is irradiated in presence of glucose and N_2O or glucose and oxygen. Here as well as in the rest of the paper, the spectra presented, when glucose was used as an additive, have been corrected for the absorption of products from glucose irradiated to the same dose under the appropriate ambient.

We have also studied the decomposition of this vitamin at different γ-doses (Fig. 7). The O. D. at 280 nm was used as a measure of concentration of the vitamin. It was observed that up to a dose of~0.4 Mrad, there is no decomposition of the vitamin when irradiated in presence of glucose and oxygen or glucose and N_2O. At the same dose the vitamin solution irradiated as such in air showed more than 50% decomposition. At the sterilization dose of~2.5 Mrad, still about 50% of the vitamin was left behind when we used the above additives, whereas without the additives there was almost complete decomposition. The degradation observed after 0.4 Mrad could be due either to depletion of the e_{aq}^- scavenging component of the system (N_2O or O_2) or due to H atom reaction with the vitamin. Further work is in progress to confirm this.

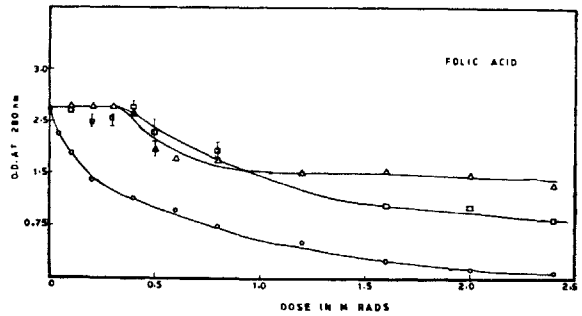


FIG. 7. Degradation of 10^{-4}M folic acid solution ($pH=6.8$). o—γ-irradiated in air; △—γ-irradiated in presence of O_2 saturated 0.1M glucose (blank 0.1M glucose, O_2 saturated); □—γ-irradiated in presence of N_2O-saturated 0.1M glucose (blank, 0.1M glucose, N_2O-saturated).

Pantothenic acid

$$CH_2OH.C.(CH_3)_2.CHOH.CONH.CH_2.COOH$$

Pulse radiolysis studies of this vitamin have been carried out by Moorthy and Hayon (*in press*). The reactivity of this compound with e_{aq}^- is much lower whereas its reactivity with OH radicals is quite high (see Table I). Hence it reacts essentially with OH radicals which can abstract an H atom from various sites in the molecule to give a number of free radicals. The sites can be C_2, C_3, C_6 and C_8. Pantothenic acid on γ-irradiation undergoes considerable changes as can be seen from Fig. 8 which shows the spectrum of the product of OH radical reaction with pantothenic acid. In presence of 0.1 M glucose and N_2O or O_2, the spectrum remains unchanged even after irradiation.

Pantothenic acid was also irradiated in presence of 0.1M glucose and air for different doses. The spectrum of the irradiated compound showed no change up to ~1 Mrad dose. At still higher doses, there was destruction of the vitamin. These results are shown in Fig. 9. In the case of pantothenic acid it may be noted that the

degradation products absorb more strongly than the parent and hence there is a build up of the absorbance of the solution with dose.

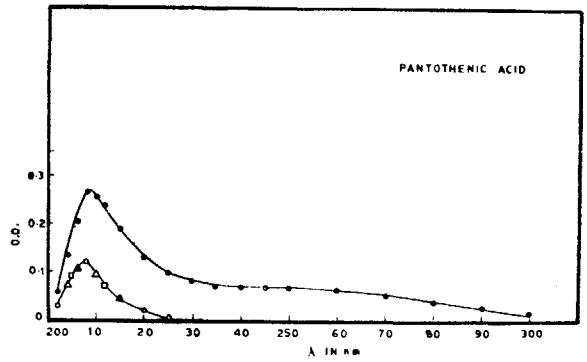


FIG 8 Absorption spectra of 10^{-4}M pantothenic acid solution ($pH = 6.8$); o-unirradiated; ●—γ-irradiated in presence of N_2O; △—γ-irradiated in presence of O_2 and 0.1M glucose; □—γ-irradiated in presence of N_2O and 0.1M glucose (spectrum corrected for the absorption of irradiated glucose solution); ▲—γ-irradiated in presence of N_2 and 0.1M glucose (spectrum corrected for the absorption of irradiated glucose solution)

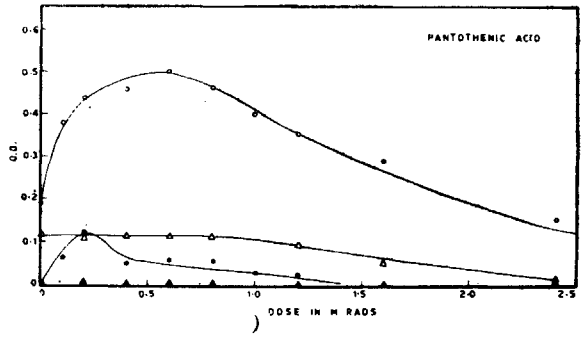


FIG 9. Degradation of 10^{-4}M pantothenic acid solution irradiated in air ($pH = 6.8$). o—O.D. at 208nm; ●—O.D. at 260 nm; △—O. D. at 208 nm 0.1M glucose is also present (blank, 0.1M glucose); ▲—O D. at 260 nm when when 0.1M glucose is also present (blank 0.1M glucose).

The other vitamins studied viz. pyridoxine, nicotinamide and riboflavin behaved in the same manner as discussed above. The results are shown in Figs. 10–12. In all cases on irradiation in presence of air or N_2O, there was considerable damage, due essentially to reaction with OH radicals. In presence of N_2 and 0.1 M glucose also there were changes in absorption spectra. Under this condition only e^-_{aq} reaction with vitamin is possible and the spectral changes presumably reflect the formation of reduction products. But in presence of glucose (0.1 M) and N_2O or oxygen, the spectra after correction for absorption by the glucose products, were unchanged as compared to the unirradiated solutions. More detailed work to establish the degradation as a function of dose is in progress. Nevertheless at least at low doses, glucose at a concentration of 0.1 M and in presence of O_2 or N_2O, is definitely functioning as a protective additive for all the

vitamins studied. It is to be noted that the protection of the vitamins by glucose is manifested only in presence of an electron scavenger such as N_2O or O_2 (in all cases except pantothenic acid), because these vitamins are reactive towards both e^-_{aq} and OH radicals. On the other hand, in the absence of an electron scavenger (as in N_2 saturated solutions) glucose does not confer protection. In the case of pantothenic acid, however, the presence of an electron scanvenger is not essential because of the rather low reactivity of this vitamin with e^-_{aq}.

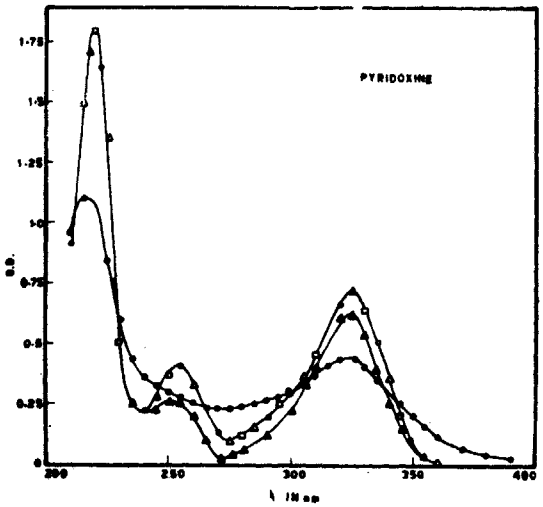


FIG. 10. Spectral changes in 10^{-4}M pyridoxine solution (pH=6.8). o–unirradiated; ●—γ-irradiated in presence of N_2O; △—γ-irradiated in presence of 0.1M glucose and O_2; □—γ-irradiated in presence of 0.1M glucose and N_2O (spectrum corrected for the absorption of irradiated glucose solution); ▲—γ-irradiated in presence of 0.1M glucose and N_2 (spectrum corrected for the absorption of irradiated glucose solution).

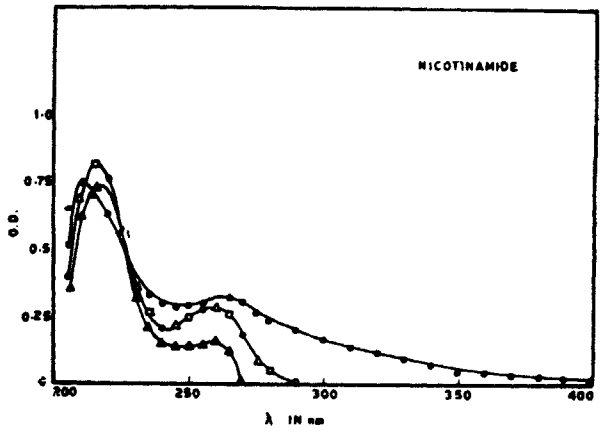


FIG. 11. Spectral changes in 10^{-4}M nicotinamide solution (pH=6.8). o—unirradiated; ●—γ-irradiated in presence of N_2O; △—γ-irradiated in presence of O_2 and 0.1M glucose; □—γ-irradiated in presence of N_2O and 0.1M glucose (spectrum corrected for the absorption of irradiated glucose solution); ▲—γ-irradiated in presence of N_2 and 0.1M glucose (spectrum corrected for the absorption of irradiated glucose solution).

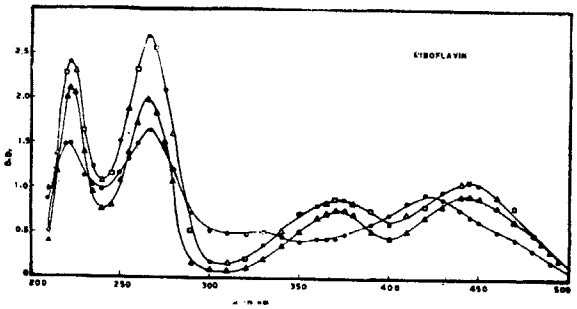


FIG. 12. Spectral changes in 10^{-4}M Riboflavin solution (pH = 6.8). o—unirradiated; ●—γ-irradiated in presence of N_2O, △—γ-irradiated in presence of O_2 and 0.1M glucose; □—γ-irradiated in presence of N_2O and 0.1M glucose (spectrum corrected for the absorption of irradiated glucose solution); ▲—γ-irradiated in presence of N_2 and 0.1M glucose (spectrum corrected for the absorption of irradiated glucose solution).

We have derived our present conclusions regarding the radiation protection of vitamins from the uv spectra of the irradiated vitamin solutions. It is proposed to substantiate these conclusions employing other methods of analysis such as polarography, thin layer chromatography and microbiological assay.

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