XA7905926

REPORT NO. IAEA-R-1688-F

TITLE

Studies on the radiation chemistry of biomolecules in aqueous solution with specific objective of minimizing their radiolytic degradation, (coord.progr. for Asia and the Pacific Region on radiation sterilization practices significant to local medical supplies and conditions)

FINAL REPORT FOR THE PERIOD

1975-11-01 - 1078-10-31

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INTERNATIONAL ATOMIC ENERGY AGENCY

DATE January 1979

Final Progress Report - IAEA

Contract No.1688/RB

August 1, 1977 to November 30, 1978

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FINAL PROGRESS REPORT

IAEA Contract No. 1688/RB

A.	i) Contract No.	:	1688/RB
	ii) Title of Project	:	Studies on the Radiation Chemistry of Biomolecules in Aqueous Solutions with the Specific Objective of Minimising their Radiolytic Degradation.
	iii) Institute where the research is being carried out	:	Bhabha Atomic Research Centre (Govt. of India, Department of Atomic Energy), Trombay, Bombay 400085.
	iv) Chief Scientific Investigator	:	Dr K. Narayana Rao
	v) Time period covered	:	August 1, 1977 to November 30, 1978.

B. Description of research being carried out:

B.(i) Objective:

Gamma-radiation is increasingly coming into use in place of conventional agents such as heat and ethylene oxide for sterilization of medical products including pharmaceutical preparations. However, before this new technique becomes acceptable, it is absolutely essential to establish that radiation does not introduce toxic substances by transformation of either the active material or the medium in which it is present during radiation sterilization. Many pharmaceutical preparations, e.g., vitamins and antibiotics are often administered in dilute aqueous media, such as syrups and suspensions. In such systems, the ionizing radiation interacts almost exclusively with

water to give hydrated electrons, hydrogen atoms, and hydroxyl radicals, and also molecular hydrogen and hydrogen peroxide. Of these, the first three are very reactive towards many functional groups present in organic molecules that constitute the active component of the pharmaceutical. Hydrogen peroxide, being capable of acting both as an oxidising and reducing agent, can cause damage to the active component. Even in the pure state a compound can undergo radiation damage as a result of ionisation and excitation events resulting from exposure to the ionizing radiation. Therefore, during radiation sterilization, pharmaceutical preparations may not only lose their potency wholly or partly but new substances would be produced whose effect on the organism could be entirely different from that of the parent. It is therefore very essential to first assess the extent of radiation damage to the active component and identify the products formed. Secondly knowing the reaction pathways that lead to the damage, it should be possible, by the addition of suitable chemicals or otherwise, to find out ways and means to minimise such radiolytic transformations.

B.(ii) Plan of work:

Bearing the above objectives in mind, the work has been organized in the following manner:

1. Qualitative study of radiolytic changes in aqueous solutions of biomolecules under well defined conditions.
2. (a) Quantitative assessment of overall damage to bio-

molecules in aqueous solution over a range of doses extending upto the conventional sterilization dose of 2.5 M rads.

(b) Quantitative assessment of overall damage to biomolecules in aqueous solution containing protective additives, over a range of doses upto 2.5 M rads.

3. Effect of variation of the concentrations of the biomolecule and the protective additives on the extent of damage of the former.
4. Effect of parameters such as pH, state of aggregation and temperature on the radiation damage to the biomolecules in aqueous solution.
5. Estimation of H_2O_2 concentration in the irradiated systems.
6. Build up of free radical concentration with dose and radical stability in biomolecules in the dry solid state - quantitative assessment of radiation damage.
7. Separation and identification of radiolytic products in irradiated biomolecules both in the dry solid state and in aqueous solutions.
8. ESR study of radical formation in frozen aqueous solutions of biomolecules.
9. Basic radiation chemistry of the biomolecules in aqueous systems.
10. Study of reaction of hydrogen peroxide with the biomolecules in aqueous solution.

11. Biochemical studies on the irradiated systems.

Part I: Report for the current period:

Out of the items detailed under B(ii), 9 and 10 have been taken up and the researches done are described below. The biomolecules covered in this investigation are the B-group vitamins: nicotinamide, pyridoxin, riboflavin and thiamine. The detailed mechanistic study pertains only to the last of these compounds.

B.(iii)(a) Experimental procedure:

All the vitamins used were the purest commercially available, supplied by M/s. Calbiochem or Sigma Chemicals. Other chemicals were either BDH AnalaR grade or E. Merck G.R. grade. Solutions were prepared in triply distilled water using phosphate buffer (10^{-2}M monobasic and 10^{-2}M dibasic phosphates), HCl or H_2SO_4 to maintain the desired pH. Irradiations were carried out in an indigenously fabricated Gamma Cell of Cobalt-60 at a dose rate of 0.35 M rad per hour as measured by Fricke dosimeter. Optical densities were read on a Beckman DU spectrophotometer. For polarographic analyses, and electrolytic reduction at a mercury pool electrode, a Bruker Modular polarograph (Model 310) was employed. The differential pulse mode was employed to realise the highest sensitivity and resolution in the assay of irradiated solutions. As discussed in the IV report, addition of 0.04% Triton X-100 to the irradiated solution greatly facilitated the differential pulse

polarographic assay in the case of thiamine. Cyclic voltammetry to identify some of the products in irradiated thiamine solutions was also carried out employing the Bruker polarograph. Chemical reduction of thiamine was carried out by using $LiAlH_4$ in anhydrous THF and extracting the dihydrothiamine into absolute ethanol /Ref.1/. Spectrofluorimetric analyses were carried out employing an Aminco Bowman Spectrofluorimeter, Model SPF-4-8202. For mass spectrometry VG Micromass model 7070F mass spectrometer was employed.

B.(iii)(b) Results and Discussion:

1. Rate constant for reaction of thiamine with primary radiolytic species: From previous pulse radiolysis studies /Ref.2/ the bimolecular rate constants for the reaction of e^-_{aq} and OH radicals with thiamine are known to be 3.4×10^{10} and 3×10^9 $dm^3 mol^{-1} s^{-1}$ respectively. The rate constant for reaction with H-atoms has now been evaluated by competition with formate and found to be 2×10^{10} $dm^3 mol^{-1} s^{-1}$ (assuming $k_{H + HCO_2^-}$ to be 2.2×10^8 $dm^3 mol^{-1} s^{-1}$).

2. Mechanistic study of thiamine radiolysis in aqueous solution: Employing differential pulse polarography (in presence of 0.04% Triton X-100) to assay thiamine in irradiated solutions, the effect of different variables such as thiamine concentration, dose, O_2 and other scavengers for the primary species on the G-value for thiamine decomposition was investigated. The results of these detailed studies have been submitted for publication and the copies of these two papers are appended. The important

findings are as follows.

Under conditions such that direct scavenging of molecular H_2O_2 by thiamine is insignificant, hydrogen peroxide yield in deoxygenated thiamine solutions is close to zero. This has been explained by postulating that the thiamine radical (Thm) formed by e^-_{aq} and H atom reaction has long enough life time to react with molecular H_2O_2 according to:

$$Thm + H_2O_2 \longrightarrow Thm^+ + OH + OH^- \quad .. \quad (1)$$

In agreement with this, the initial yield for thiamine destruction in the absence of O_2 is 6 and corresponds to the stoichiometry

$$G(-Thm^+) = g_{e^-_{aq}} + g_H + g_{OH} \quad .. \quad (2)$$

In oxygen saturated solutions the measured H_2O_2 yield (= 2.6) and thiamine destruction (G = 1.2) corresponded to the stoichiometry

$$G(H_2O_2) = g_{H_2O_2} + \tfrac{1}{2}(g_{e^-_{aq}} + g_H) \quad .. \quad (3)$$

and $$G(-Thm^+) = \tfrac{1}{2}gOH \quad .. \quad (4)$$

From this it is inferred that the radicals formed by reaction of OH with thiamine undergo disproportionation in presence of O_2:

$$Thm^+ + OH \longrightarrow Thm(OH)^+ \xrightarrow{O_2} Thm(OH)(O_2)^+ \quad .. \quad (5)$$

$$2Thm(OH)(O_2)^+ \longrightarrow Thm^+ + P^+ + 2O_2 \quad .. \quad (6)$$

where P^+ is a product. Under conditions such that electrons are scavenged by Thm^+ and not O_2, it was observed that H_2O_2 yield corresponded to the stoichiometry of equation (3). This would

indicate that the thiamine radicals formed by e^-_{aq} and H atom reaction can reduce O_2 to H_2O_2 via:

$$Thm + O_2 \longrightarrow Thm^+ + O_2^- \quad .. \quad (7)$$

and $$O_2^- + HO_2 \xrightarrow{H^+} H_2O_2 + O_2 \quad .. \quad (8)$$

Reaction (7) is in agreement with a value of -0.5 V for the reversible one electron redox potential of thiamine determined by redox titration of thiamine with various radiolytically generated radicals of known one electron redox potential.

3. Radiolytic reduction product of thiamine: Radiolytic reduction of thiamine was carried out via reaction (9) - (12)

$$e^-_{aq} + N_2O \xrightarrow{H_2O} N_2 + OH + OH^- \quad .. \quad (9)$$

$$OH + (CH_3)_2CHOH \longrightarrow (CH_3)_2C(OH) + H_2O \quad .. \quad (10)$$

$$(CH_3)_2C(OH) + Thm^+ \longrightarrow (CH_3)_2CO + Thm + H^+ \quad .. \quad (11)$$

$$Thm \longrightarrow product \quad .. \quad (12)$$

The resulting product on TLC separation (Silica gel G/$CHCl_3$: EtOH:H_2O) exhibited the same R_f value (0.80) and UV spectrum (λ_m = 237 nm and 280 nm) as the one obtained by electrolytic reduction at a mercury pool electrode at -1.3 volts vs SCE. In the EI mode mass spectrum of both, there was a parent peak at m/e = 267 indicative of dihydrothiamine (DHT). However, the polarographic behaviour of the two were different - the electrolytic reduction product exhibited an anodic wave at $E_{\frac{1}{2}}$ 0.4 V vs SCE while the radiation chemical product exhibited a cathodic wave at the same $E_{\frac{1}{2}}$. To resolve this difficulty, chemical reduction of thiamine was carried out [Ref.17] employing Li AlH_4

as the reducing agent. DHT is known to exist in three isomeric forms: normal (n-), iso(i-) and pseudo (p-) [Ref.3]. In absolute ethanol the absorption spectrum of p-DHT is distinctly different from those of the n- and i- forms [Ref.4]. In aqueous solution the latter exhibit an anodic polarographic wave at -0.4 V vs SCE while the p-isomer exhibits only a cathodic wave at the same potential [Ref.3]. Comparison of both the classical polarograms and cyclic voltammograms in aqueous solution and the absorption spectra in absolute ethanol revealed that whereas chemical reduction according to the above procedure gave exclusively the p-DHT while the other two methods gave mixtures of the three forms, the n- and i- DHT being present in much greater proportion in the electrolytic product.

4. Reaction of H_2O_2 with the vitamins in aqueous solution: As H_2O_2 is one of the radiolytic products when aqueous solutions are irradiated in presence of oxygen, its effect on the vitamin content of solutions was investigated. Solutions of known concentrations of H_2O_2 and the vitamin were mixed and the concentration followed periodically in comparison with a blank which did not contain H_2O_2. The results are given in Table 25. With all the vitamins the change in concentration was no more than in the controls not containing H_2O_2. In fact over the time period monitored the loss of H_2O_2 by catalytic decomposition is appreciable. It is therefore concluded that the loss in potency of the irradiated vitamin solutions due to reaction with radiolytically produced H_2O_2 is negligible.

Conclusions of the study under the Project - 1688:

The radiolytic degradation of four molecules of the vitamin B-complex group, viz., nicotinamide, pyridoxin, riboflavin and thiamine have been investigated in detail. The various aspects studied were - radiolysis under controlled conditions, effects of phase, temperature, pH and nature and concentration of additives. Apart from gaining insight into the basic mechanism of radiolytic breakdown of these molecules, the aim of the study was to find out irradiation conditions under which the radiolytic degradation can be minimised. A number of analytical techniques have been employed for the quantitative assay of undamaged vitamin in the irradiated material and identification and assay of products formed. These included spectrophotometry, spectrofluorimetry, TLC separation followed by one or both of the above methods, polarography, mass spectrometry and ESR. One outgrowth of this work has been the development of a differential pulse polarographic procedure for thiamine assay which is not subject to interference by its radiolytic products. In general polarographic procedures requiring no preliminary separation and hence simpler for routine work, were found to give results comparable to those obtained by the more authentic but cumbersome procedure of TLC separation and assay by spectrophotometry.

The results of the various investigations carried out under this programme reveal the following:

1. With the oxygen saturated aqueous vitamin solutions containing glucose the radiolytic degradation of the vitamin is

considerably reduced, the extent of such protection depending on such factors as the concentrations of vitamins, glucose, dissolved oxygen and radiation dose. The results are explicable on the basis of the known rate constants for reaction of the various molecules present in the system with the primary radiolytic species from water.

2. The observations in N_2O saturated aqueous vitamin solutions are closely similar to those in (1) above.

3. In glucose containing solutions the protective effect is considerably modified at higher irradiation temperatures. Thus, e.g., at 68°C, the protection is markedly enhanced in the case of nicotinamide, pyridoxin and riboflavin, but retarded in the case of thiamine.

4. Irradiation of air saturated aqueous solutions in the frozen state (ice) leads to reduced decomposition, the reduction being more the lower the temperature. At -80°C (dry ice bath) for example, the extent of damage is less than 10% even at 2.5 M rads; this can be further reduced by the addition of glucose.

5. At the sterilization dose of 2.5 M rads the stationary concentration of radiolytically formed H_2O_2 is higher in the case of irradiation in the frozen matrix at -80°C than in the case of fluid solution at 27°C.

6. The thermal reaction of H_2O_2 with the vitamins is rather slow. As the steady state concentration of H_2O_2 in solutions irradiated to the sterilization dose is 10^{-5} molar, the

contribution of this to post irradiation destruction of vitamins can be concluded to be negligible.

7. Irradiation of the vitamins as dry solids leads to no damage detectable on dissolution in water although the steady state trapped radical concentration formed at 2.5 M rads is 0.1%.

8. In the case of thiamine the product of hydrated electron reaction has been shown to be the same as the chemical reduction product viz., dihydrothiamine. This may also be true of the other vitamins since in all cases the first step is a one electron reduction as revealed by earlier pulse radiolytic studies [Ref.4].

9. The product of reaction of OH radicals with thiamine is not the same as obtained by chemical oxidation, viz., thiochrome. This may be attributable to the fact that OH radical reactions can occur at many sites in the molecule and can add on to the hetero aromatic rings or abstract hydrogen from side chains etc., and very rarely a one electron oxidation. This may be true of other vitamins also. In all cases, however, a number of products are formed.

The importance of some of these findings in connection with the radiation sterilization of pharmaceutical preparations is evident. Sugars, although not necessarily glucose, are used as carriers in all such syrupy preparations. In presence of oxygen, and particularly in frozen matrices at low temperatures, it appears possible to reduce the radiolytic breakdown of the vitamins to low levels. From the published literature it is known that the products of glucose radiolysis, although harmful

towards micro organisms, are non-toxic to mammals [Ref.5]. Further, such products would be formed in much lower yield when irradiation is carried out in the frozen matrix at low temperature. This is much superior to irradiation at higher than ambient temperature, though higher irradiation temperature is beneficial from the point of view of killing the micro-organisms because a reduction in the sterilization dose is possible due to the synergistic effect of heat and radiation [Ref.6]. Although micro-organisms are known [Ref.7] to be less sensitive to radiation at low temperatures, as the indirect effect contribution originating from water is reduced, direct effect, which is about 50% of the total should still be effective and hence perhaps a higher sterilization dose may be required at the lower temperature. From the results of the present study it can be inferred that even at such higher doses, the radiolytic damage to the vitamins in the frozen matrix at -80°C could be low enough to be acceptable.

Perhaps the best method of sterilization of pharmaceutical preparations would be to irradiate the components in the dry solid state and compound them together after irradiation. If this should not be feasible for practical reasons, then the next best procedure is irradiation in the frozen matrix in a dry ice bath.

These conclusions may be applicable to other pharmaceuticals also.

References:

1. G.E. Bonviani and D.J. Hennessy, J.Amer.Chem.Soc., 79, 6325 (1957)

2. P.N. Moorthy and E. Hayon, J.Org.Chem., 42, 879 (1977)

3. K. Okamoto, Rev. Polarography (Japan), 12, 50 (1964)

4. P.N. Moorthy, E. Hayon and E.J. Hart, Intern.Conf. on Radiation Chem., Argonne, USA, 1975; paper III.7

5. J. Schubert, Proceedings of the V Intern.Congr.Rad.Res., Seattle, USA, 1974, Paper E-17-4

6. C.A. Trauth and H.D. Sivinski, "Radiation sterilization of medical products", Proceedings of IAEA Symposium, Bombay 1974

7. T. Sanner and A. Pihl, Rad.Res., 37, 216 (1969)

TABLE 25

Effect of H_2O_2 on the stability of vitamin solutions
10^{-4}M vitamins in presence of 4.5×10^{-4}M H_2O_2 pH 6.8

Time (days)	% decomposition of thiamine		% decomposition of riboflavin		% decomposition of pyridoxin		% decomposition of nicotinamide		% decomposition of H_2O_2
	Alone	In presence of H_2O_2	Alone	In presence of H_2O_2	Alone	In presence of H_2O_2	Alone	In presence of H_2O_2	
1	3.2	3.1	0	0	5.0	5.0	13.0	13.0	50
2	6.4	6.1	0.8	0.8	7.0	7.0	20.0	20.0	60
4	9.0	9.1	1.6	1.6	8.3	8.3	25.0	25.0	90

KMR:
7.(1.1

Publications arising from the Research Contract:

1. Kamal Kishore, P.N. Moorthy and K.N. Rao, Radiat.Effects, 27, 167 (1976). "Radiation protection of vitamins in aqueous systems - Part I".

2. Kamal Kishore, P.N. Moorthy and K.N. Rao, Radiat.Effects, 29, 165 (1976), "Radiation protection of vitamins in aqueous systems - Part II . A comparative study in fluid and frozen aqueous systems".

3. Kamal Kishore, P.N. Moorthy and K.N. Rao, Radiat.Effects, In press. "Radiation protection of vitamins in aqueous systems. Part III. Effect of different variables".

4. Kamal Kishore, P.N. Moorthy and K.N. Rao, Ind.J.Chem. In press. "Thiamine assay by differential pulse polarography".

5. P.N. Moorthy, K.N. Rao and Kamal Kishore, Radiat.Effect Letters. In press. "Redox potential of thiamine".

6. Kamal Kishore, P.N. Moorthy and K.N. Rao, Radiat.Phys. Chem. (submitted). "Radiation induced reactions in aqueous thiamine (vitamin B_1)".