

Activity of radiation degradation products of vitamins A and E to haemolyse erythrocyte

BRIJ BHUSHAN, V. NINJOOR and G. B. NADKARNI*

Biochemistry and Food Technology Division, Bhabha Atomic Research Centre, Trombay, Bombay 400085, India

Abstract. Exposure of vitamin A acetate in freely dissolved state to γ -radiation *in vitro* caused a dose dependent degradation accompanied by the formation of new products. The radiation degradation products were separated by chromatography using step gradient elution. The parent molecule, vitamin A acetate, induced negligible haemolysis of erythrocytes. In contrast, the polar products formed by irradiation were found to be potent haemolysing agents. A highly polar product, eluted with methanol revealed maximum haemolytic activity. Acetylation of these products resulted in loss of their haemolytic properties. Similarly, vitamin E acetate, a known stabilizer of the biomembranes, after irradiation yielded products which caused haemolysis of erythrocytes. It was demonstrated that irradiation introduces hydroxyl groups which impart haemolytic properties to the radiation degradation products of vitamin A.

Keywords. Vitamin A; vitamin E; radiation degradation products; erythrocyte haemolysis.

Introduction

Efficacy of vitamin A alcohol (retinol) in causing membrane damage *in vitro* leading to haemolysis of erythrocytes (Seeman, 1970; Dingle and Lucy 1962), swelling of mitochondria (Keiser *et al.*, 1964; Goodall *et al.*, 1980) and release of hydrolases from lysosomes (Roels 1969) has been well documented. In addition to causing membrane perturbation, vitamin A is also believed to be involved in maintaining the structural integrity of biomembranes (Mack *et al.*, 1972; Dingle and Lucy, 1962). The aspects relating to the stabilizing and labilizing activity of vitamin A become more pertinent in view of the observed fragility of erythrocytes in hypo and hyper-vitaminosis (Seeman 1970). Several studies (De Luca *et al.*, 1970; Mack *et al.*, 1972; Glover *et al.*, 1974) pointing to the incorporation of injected labelled retinol in the cellular membranes further support the suggestion that vitamin A is involved in the regulation of biomembrane stability. However, derivatives and structural analogues of vitamin A are reported to be weak in their membrane damaging potential presumably due to the modifications affected in either β -ionone ring or conjugated double bonds or in the terminal polar group (Seeman 1970; Perumal *et al.*, 1968; Dingle and Lucy, 1962). In contrast, our earlier studies have demonstrated that exposure of vitamin A to γ -

* To whom correspondence should be addressed.

Abbreviations used: TLC, Thin layer chromatography; PBS, phosphate buffer saline 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.0; SDS, sodium dodecyl sulphate; Gy, gray; UV, ultra-violet; IR, infrared; NMR, nuclear magnetic resonance; MS, mass spectrum.

radiation leads to the formation of polar radiation degradation products and these are involved in the labilization of lysosomes (Ninjoor *et al.*, 1972; Bhushan *et al.*, 1977).

The present communication reports on the interaction of radiation degradation products of vitamin A with simpler membrane system such as erythrocytes. The data seem to suggest that acquisition of hydroxyl groups following irradiation of vitamin A imparts labilizing activity to these products.

Materials and methods

Pure crystalline vitamin A acetate was a gift from Roche Products (India) Ltd., Bombay. Vitamin A alcohol was prepared by saponification of vitamin A acetate. DL α -tocopherol acetate (vitamin E) was obtained from E. Merck, Darmstadt, Germany and Duponol 'C', a mixture of sulphated alcohols of varying chain lengths from Du Pont, Newtown, Connecticut USA. Eicosanol (arachidyl alcohol) was procured from Fluka, Switzerland. Surface active agents; Brij-35 (polyoxyethylene-23-lauryl ether), saponin, sodium dodecyl sulphate (SDS), Triton X-100 (*p*-isoocty-phenoxyethoxy ethanol) and emulsifiers Tween-80 (polyoxyethylene sorbitan monooleate) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. The spectrophotometric alcohol was prepared by distillation of ethanol treated with CaO (10 % w/v), NaOH (2% w/v) and zinc dust (Henick *et al.*, 1954). Petroleum ether 60-80 from British Drug House (India) Ltd., Bombay was distilled before use.

Erythrocytes

The rabbits housed in the animal facility of this Research Centre were bled from the marginal ear vein and the blood was collected in Alsevier's solution (De Gowin *et al.*, 1949). The erythrocytes were separated according to a modified procedure of Wintrobe (1974). The blood was centrifuged at 5 *g* for 10 min and washed twice with 10 volumes of 0.15 M NaCl solution. The cells were resuspended in phosphate buffer saline (PBS, 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.0) and centrifuged at 50 *g* for 10 min in haematocrit tubes. The packed cell volume was determined and 2 % cell suspension in PBS was prepared for haemolysis experiments.

Irradiation of vitamins and separation of radiation degradation products

Acetate derivatives of vitamins A and E dissolved in non-polar lipid solvent, petroleum ether (1 mM) were exposed to varying doses of γ -radiation upto a dose of 15 kGy in Gamma Cell-220 (AECL, Canada) at a dose rate of 5.6 kGy/h with air as gas phase. For the separation of the degradation products, only the samples exposed to 10 kGy were chosen. Step gradient elution chromatographic technique described earlier (Ninjoor *et al.*, 1972) was followed to separate the products. Briefly, the procedure consisted of flash evaporation of the solvent and loading the residue dissolved in 0.5 ml of petroleum ether on a glass column (30 \times 1.5 cm) packed with 6 % (v/w) water deactivated alumina to a height of 10 cm and sequential elution of the products with 25 ml each of following solvents: petroleum ether and petroleum ether containing 5, 20 and 50 % diethyl ether.

Highly polar radiation degradation product of vitamin was eluted with methanol. The eluted fractions were subjected to thin layer chromatography (TLC) using silica gel G as support and 8 % acetone in petroleum ether as solvent system.

The products from irradiated vitamin E acetate were separated similarly on 6 % (v/w) water deactivated alumina column. Only two product were isolated from irradiated vitamin E acetate. Polar radiation degradation products was eluted with 50 % diethyl ether in petroleum ether while highly polar products required methanol for their elution.

The products separated by column chromatography were analysed for their ultra-violet (UV) and infrared (IR) absorption pattern and the products with hydroxyl groups were acetylated (Stahl, 1969). The acetylated derivatives were extracted with diethyl ether and purified by chromatography on alumina column employing 5% diethyl ether in petroleum ether as eluent.

Haemolytic activity

The haemolytic activity (Wessels *et al.*, 1963) of the radiation degradation products was determined by treating the erythrocyte suspension (2.5 ml) in PBS with test compounds dissolved in 20 μ l of spectrophotometric ethanol. The reaction mixture was vortexed and incubated at 37°C for 30 min. Erythrocytes treated with ethanol alone served as control. The reaction was terminated by adding 0.25 ml of 1.5 M NaCl and the tubes were centrifuged at 50 *g* for 10 min. The concentration of haemoglobin released was computed after recording the absorbance at 540 nm.

The concentration of the test compound required for 50 % haemolysis of erythrocytes *i. e.* C_{50} was calculated from the sigmoidal curves obtained by plotting the per cent haemolysis against the concentration of the compounds.

Results

Haemolytic activity of irradiated vitamin A acetate

Vitamin A acetate irradiated with γ -rays showed a dose-dependent degradation of the molecule and an increase in erythrocyte haemolysing activity as shown in figure 1. The disappearance of vitamin A concomitant with the increase in the haemolytic activity followed an exponential relationship. Influence of incubation period on the haemolytic activity of vitamin A subjected to varying doses of γ -radiation is depicted in figure 2. There was a direct relationship between the period of incubation at 37°C and the extent of release of haemoglobin. Irradiated vitamin A acetate released more haemoglobin than control which increased further during the progress of incubation period. The comparative haemolytic activity of irradiated and unirradiated vitamin A acetate in terms of variation in both concentration and incubation time is represented as a histogram in figure 3. Vitamin A acetate was a poor membrane labilizer since it caused less than 10% lysis even at high concentration of 1000 μ g/ml. Exposure to radiation, however rendered it into a potent labilizer as evidenced by the spurt in haemolysis. At a high concentration of 1000 μ g/ml 90% haemolysis of erythrocytes was observed. Incubation at 37°C further accentuated the extent of haemolysis at all concentrations.

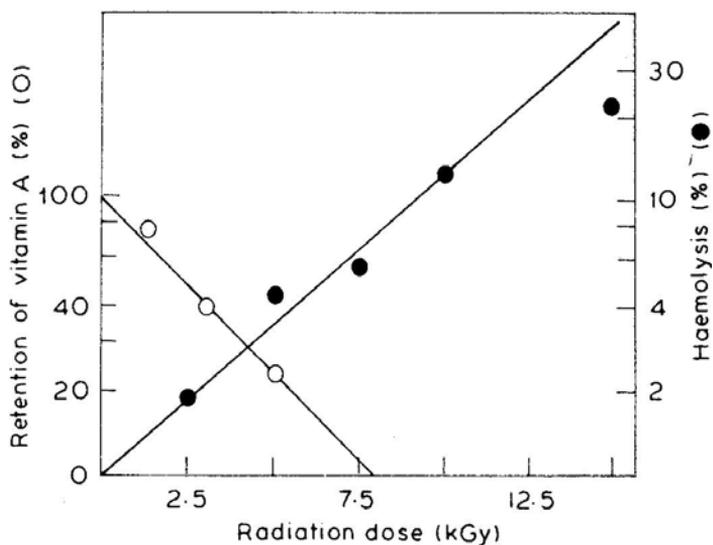


Figure 1. Dose dependent haemolytic activity of irradiated vitamin A acetate. The retention of vitamin A acetate (1 mM) was assessed spectrophotometrically following exposure to γ -radiation (0–15 kGy). Solvent was evaporated and the residue, redissolved in minimum amount of ethanol was treated with erythrocytes suspension in PBS at a concentration of 100 $\mu\text{g}/\text{ml}$. The haemoglobin released was measured spectrophotometrically. Retention of Vitamin A (O); per cent haemolysis (●).

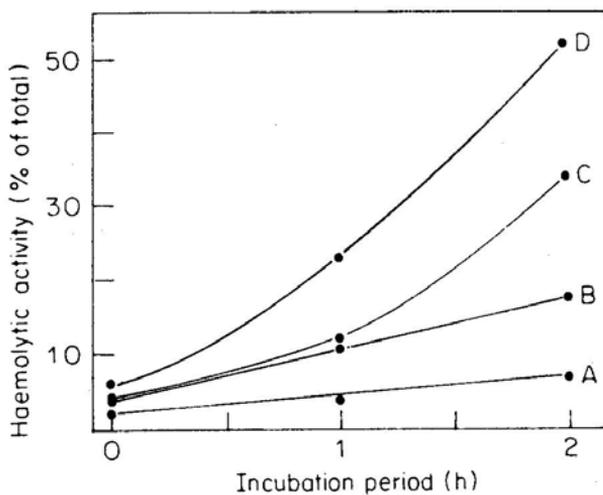


Figure 2. Effect of irradiation and incubation on haemolytic activity of vitamin A acetate. Erythrocyte suspension in PBS was treated with irradiated vitamin A acetate and incubated at 37°C for varying intervals of time. Untreated vitamin A acetate served as control. The reaction was stopped with 1.5 M NaCl and the haemoglobin released was measured as described in the text. Control (A), 5 kGy (B), 10 kGy (C), 15 kGy (D).

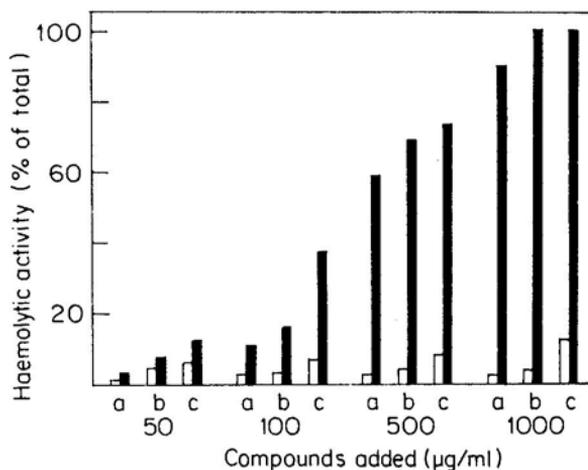


Figure 3. Effect of concentration and incubation time on haemolytic activity of irradiated vitamin A acetate. No incubation-(a); incubation (1h)-(b); Incubation (2h)-(c); (□), control; (■) irradiated 15 kGy.

Isolation of degradation products from irradiated vitamin A acetate

Vitamin A when irradiated at 10 kGy showed a sharp decline in its absorption peak at 325 nm and the emergence of a broad peak at a region of 220—260 nm which is characteristic of these products (Ninjoor *et al.*, 1978). When the products were separated as described earlier, five components tentatively named as products a, b, c, d and e could be isolated based on their polarity, R_f values, absorption characteristics and response to Carr-Price Colour reaction. Table 1 summarises the properties of these products.

Comparative haemolytic activity of radiation degradation products of vitamin A acetate

Table 2 includes the data on haemolysing potential of different degradation products. It is evident that among the products, product “e” was the most potent haemolytic agent followed by products “c” and “d”. Less polar products however, did not show significant haemolysing activity. Similarly a synthetic alcohol like eicosanol having the same number of carbon atoms as retinol failed to cause significant lysis of erythrocytes.

Figure 4 shows concentration dependent haemolytic activity of the potent compounds like products “e” and “d”. While the parent molecule showed very low haemolytic activity, the highly polar product “e” was found to be potent even at a concentration as low as 55 µg/ml for 50 % haemolysis. Under similar conditions, a known labilizer retinol was effective at 22 µg/ml to show 50 % haemolysis. The effect of product “d” was less pronounced than product “e” as indicated by a rather high C_{50} value of 500 µg/ml.

The data presented in table 3 reveal loss in haemolytic activity of products following acetylation. Thus, in contrast to high haemolytic activity exhibited by polar product “c”

Table 1. Physicochemical properties of radiation degradation products separated from the irradiated vitamin A acetate.

Compound	Eluting solvent	R_f	$\lambda_{\text{nm}}^{\text{max}}$	$E_{1\%}^{1\text{cm}}$	Colour with Carr-Price test	Yield %
Vitamin A acetate	1% Diethyl ether in PE	0.84	325	1850	Blue	—
RDPa	Petroleum ether	0.96	240	—	Green	10.2
RDPb	5% Diethyl ether in PE	0.80	240, 278*	246	Red	12.8
PDPc	20% Diethyl ether in PE	0.40	225	250	Blue	9.2
RDPd	50% Diethyl ether in PE	0.12	240, 280*	180	Violet	18.8
RDPe	Methanol	0.03	213, 245*	288	Blue-green	20.8

Vitamin A acetate (1×10^{-3} M) in petroleum ether was irradiated at a dose of 10 kGy and the products were separated by column chromatography as described in the text. The eluted fractions were freed from the solvent and subjected to TLC. The R_f values were calculated after developing the plates with 8% acetone in petroleum ether. The compounds were ascertained for their response to Carr-Price reagent (Carr and Price 1926) and absorption maxima. The $E_{1\%}^{1\text{cm}}$ was calculated on the basis of E_{max} values of the compounds at wavelength where the major peak was observed.

* Indicates inflection in the absorption spectrum.

RDP, radiation degradation product.

Table 2. Haemolytic activity of vitamin A acetate and its radiation degradation products.

Addition*	Extent of haemolysis at†			
	0'	30'	60'	120'
Ethanol	1.41 ± 0.22	2.45 ± 0.48	3.25 ± 0.15	3.99 ± 0.46
Vitamin A acetate	2.15 ± 0.21	11.41 ± 1.41	24.19 ± 9.54	30.71 ± 6.01
RDPb	5.79 ± 1.16	6.81 ± 0.69	7.69 ± 0.39	11.83 ± 0.75
RDPc	7.44 ± 2.83	50.45 ± 5.29	80.72 ± 7.44	93.10 ± 4.45
RDPd	8.90 ± 0.90	14.09 ± 1.15	27.16 ± 5.31	45.18 ± 4.14
PDPe	6.74 ± 0.88	91.35 ± 1.64	97.50 ± 1.38	99.50 ± 0.84
Eicosanol	4.50 ± 0.50	6.50 ± 1.50	6.00 ± 0.91	17.00 ± 2.22

* Compounds (100 µg/ml) dissolved in ethanol (20 µl) were added to erythrocyte suspension (2.5 ml) and incubated at 37°C for varying intervals. Radiation degradation products were separated by step gradient elution chromatography as described in the text.

† Values are mean ± SEM of 6 independent experiments.

and "e", their acetylated derivatives did not show appreciable haemolytic activity even after 2h incubation at 37 °C.

Haemolytic activity of radiation degradation products and detergents

Figure 5 presents a comparison of haemolysis caused by radiation degradation

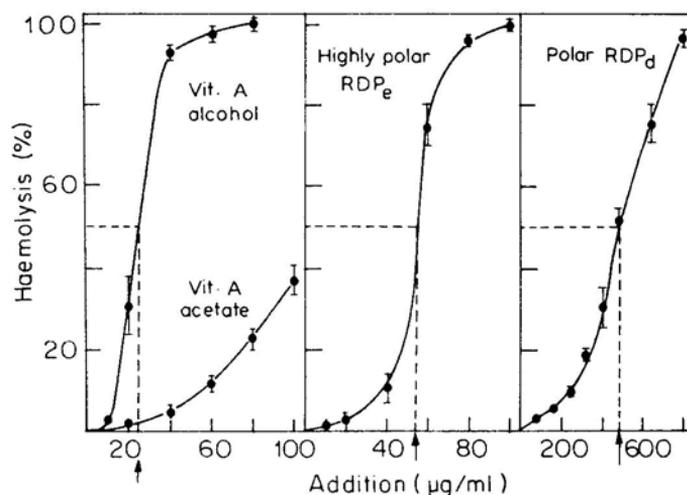


Figure 4. Concentration dependent erythrocyte lysing activity of vitamin A and its polar radiation degradation products. The erythrocyte suspension was treated with varying concentration of compounds and per cent haemolysis was measured as described in the text after incubation at 37°C for 30 min. Each point represents mean of 6 observations \pm SEM. The arrow indicates C_{50} value.

Table 3. Effect of acetylation on the haemolytic activity of the products of vitamin A acetate.

Addition*	Haemolysis (%)†			
	0'	30'	60'	120'
RDPc	7.44 \pm 2.83	50.45 \pm 5.29	80.72 \pm 7.44	93.10 \pm 4.45
Acetylated RDPc	1.57 \pm 0.23	2.03 \pm 0.27	2.75 \pm 0.72	3.41 \pm 0.75
RDPd	8.90 \pm 0.90	14.09 \pm 1.15	27.16 \pm 5.31	45.18 \pm 4.14
Acetylated RDPd	1.49 \pm 0.18	2.05 \pm 0.24	3.57 \pm 0.61	5.42 \pm 1.18
RDPe	6.74 \pm 0.88	91.35 \pm 1.64	97.50 \pm 1.38	99.50 \pm 0.84
Acetylated RDPe	2.48 \pm 0.26	1.39 \pm 0.33	1.62 \pm 0.23	3.24 \pm 1.39

* Compounds (100 μ g/ml) dissolved in ethanol (20 μ l) were added to erythrocyte suspension (2.5 ml) and incubated at 37°C for varying intervals. Acetylation of products was carried out with acetic anhydride and pyridine as described in the text,

† Values are mean \pm SEM of 6 experiments.

products of retinyl acetate with the known detergents. It was observed that treatment of erythrocyte suspension with non-ionic detergents such as Triton X-100, Brij 35 and Saponin at a concentration of 100 μ g/ml failed to elicit release of haemoglobin, whereas product "c" and "e" produced 50 and 100% lysis respectively. Ionic detergents such as Duponol and SDS at the same concentration resulted in 70% cell lysis, which is more than that obtained by product "c" but less than product "e". In order to achieve comparable haemolytic activity as affected by products, it was necessary to increase the concentration of non-ionic detergents ten fold.

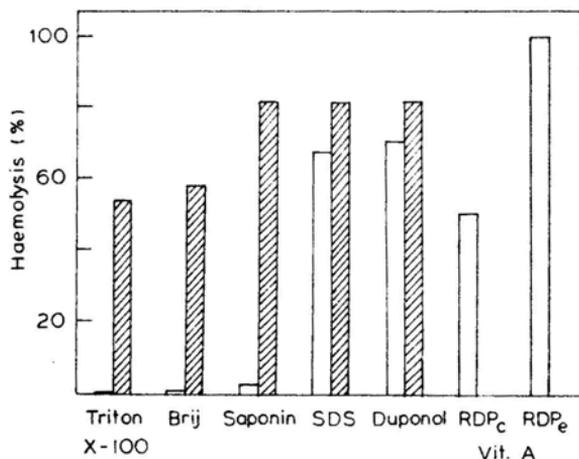


Figure 5. Comparative erythrocyte lysing activity of surface active agents and polar products of vitamin A acetate. Erythrocyte suspension in PBS was treated with polar products of vitamin A acetate and surface active agents in 20 μ l ethanol at 37°C for 30 min. Per cent haemolysis was measured as described in the text. Tween-20, Tween-80, polyethylene glycol, sodium thioglycolate, sodium barbitone and sodium tripolyphosphate were found to be ineffective, (\square), 0.1 mg/ml; (hatched), 1.0 mg/ml

Radiation induced haemolytic activity of vitamin E acetate

Treatment of erythrocytes with vitamin E acetate at a concentration of 100 μ g/ml did not result in any haemolysis even after 4 h incubation at 37°C (table 4). However, irradiated vitamin E acetate showed dose dependent increase in haemolytic activity which increased further upon incubation at 37°C.

The data incorporated in figure 6 provide a comparison of haemolytic potency of the two radiation degradation products of vitamin E acetate. The haemolytic activity of the products was observed to increase sigmoidally with the concentration. Under similar conditions, vitamin E or its acetate derivatives were inactive. Highly polar product

Table 4. Influence of irradiated vitamin E acetate on erythrocyte haemolysis.

Radiation dose (kGy)	Haemolysis* (%)			
	0 h	1 h	2 h	4 h
Unirradiated	0.55 \pm 0.17	0.70 \pm 0.25	0.83 \pm 0.22	1.25 \pm 0.28
5	1.25 \pm 0.37	2.09 \pm 0.19	4.17 \pm 0.61	3.89 \pm 0.35
10	1.11 \pm 0.17	5.97 \pm 0.82	6.53 \pm 0.76	6.39 \pm 0.18
15	5.28 \pm 0.41	14.17 \pm 2.14	19.17 \pm 4.47	28.13 \pm 7.48

Vitamin E acetate (1×10^{-3} M) in PE was irradiated at varying doses of γ -radiation. After evaporating off the solvent the resulting compounds were dissolved in 20 μ l of ethanol and added to erythrocyte suspension (2.5 ml) at a concentration of 100 μ g/ml.

* Values are the mean \pm SEM of 6 experiments.

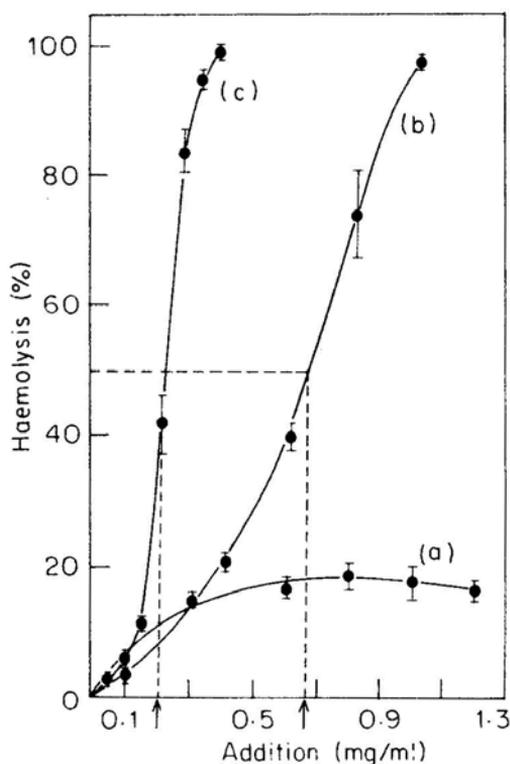


Figure 6. Comparative erythrocyte haemolysing activity of vitamin E and polar products of vitamin E acetate. Vitamin E acetate (1 mM) was irradiated with 10 kGy dose and the polar and highly polar products were separated as described in the text. Erythrocytes were treated with the products at 37°C for 30 min and haemoglobin released was measured. The points are mean of 6 observations \pm SEM. The arrow indicates the C_{50} value. Vitamin E (a), polar radiation degradation product (b), highly polar degradation product (c).

showing C_{50} value of 0.21 mg/ml was more potent in causing cell lysis than the other polar product; the corresponding C_{50} value being 0.67 mg/ml.

Discussion

Retinol is a principal biological constituent that govern the integrity of several biomembranes (Seeman, 1970; Lotan, 1980; Mullick *et al.*, 1983). In this study it is shown that the esterified forms of both vitamin A and vitamin E are transformed into potential membrane labilizers following exposure to γ -radiation. It is noteworthy that loss in vitamin A content and increase in the haemolytic activity followed a logarithmic relationship with the radiation dose apparently due to the radiation degradation products, formed as a consequence of primary event during irradiation (Bacq and Alexander, 1961). Vitamin E, often used as an antioxidant and antihaemolytic agent (Bunyan *et al.*, 1960; Fukuzawa *et al.*, 1971) also showed enhanced erythrocyte

haemolyzing activity following radiation exposure though its potency was lower than that of irradiated vitamin A and its products. When the degradation products were isolated and their haemolytic activities were compared in terms of C_{50} value, it was found that the membrane damaging potential was higher for more polar compounds. This is in accordance with our earlier observations on lamb liver homogenate (Ninjoor *et al.*, 1972) and rat liver lysosomes (Bhushan *et al.*, 1977) where the addition of polar products was shown to promote the release of hydrolytic enzymes from the lysosomal matrix.

The loss in the lytic activity of the radiation degradation products following acetylation including the highly polar product "e" suggests that hydroxyl groups are probably involved in imparting lytic potential to these compounds. The IR spectral analysis indeed confirm the presence of hydroxyl groups in these compounds as evidenced by a broad peak at 3448 cm^{-1} and its disappearance following acetylation. Additionally, despite possessing absorption maxima at lower UV regions, which is indicative of deconjugation of double bonds, the degradation products revealed lower R_f values than the parent molecule apparently due to the incorporation of polar groups. The involvement of hydroxyl groups in causing haemolysis of erythrocytes as exemplified by the haemolytic activity of aliphatic alcohols is well documented (Goodall *et al.*, 1980; Dingle and Lucy 1962). It is also established that this activity could be correlated with the increase in the length of the hydrophobic carbon chain (Seeman, 1970). However, an aliphatic alcohol having carbon chain length similar to retinol, *i.e.* phytol has been found ineffective in causing erythrocyte haemolysis (Dingle and Lucy, 1962) perhaps due to the absence of conjugated double bonds. Nevertheless, eicosanol, an archidyl alcohol resembling phytol, despite having conjugated double bonds and a terminal hydroxyl group failed to evoke haemolysis of erythrocytes as demonstrated in the present study. These observations imply that the mere presence of hydroxyl group and conjugated double bonds is not sufficient to confer lytic effect to the compound.

The erythrocyte lytic action of radiation degradation products could not therefore be ascribed to the presence of only hydroxyl groups and alternate single and double bonds. The importance of β -ionone ring in biological activity and lytic effects of vitamin A is well established (Lotan, 1980; Dingle and Lucy, 1962). Our observations on the detailed structure of the products (to be published elsewhere) as deduced by IR, NMR and MS data show that exposure of vitamin A acetate to γ -radiation leaves the ring structure intact, but modifies the side chain. The modifications include the incorporation of hydroxyl groups and deconjugation of double bonds.

As regards the mechanism of membrane disruption by radiation degradation products of vitamin A the interpretation could only be based on the findings from the studies on the known haemolytic agent, retinol. As a probable hypothesis for retinol mediated cell lysis it has been suggested that the action is at a site on the lipoprotein membrane wherein the methyl groups of the vitamin A bind to membrane cholesterol by Van der Waals forces, while the hydroxyl group is attached to membrane protein by hydrogen bonding (Glauert and Lucy, 1968). The sequential changes in erythrocyte membrane actively interacting with retinol have been documented by electron microscopic studies (Glauert *et al.*, 1963). These changes comprise of appearance of large indentations, formation of vacuoles followed by emergence of nicks in membrane leading to the release of haemoglobin. Further, the crucial factor in the efficacy of

alcohols to lyse erythrocyte membrane seemed to depend on their hydrophobicity (Kondo and Kasai, 1973). Studies with several labilizers of biomembranes have shown that their potency may be correlated with the existing balance between the hydrophilic and hydrophobic regions in their structure (Seeman, 1970). In view of these findings it could be suggested that the observed increase in haemolyzing activity of polar products of vitamins is due to the enhanced hydrophilicity of the compounds mainly as a consequence of acquisition of hydroxyl groups during exposure to γ -radiation.

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