

## VITAMIN A-INDUCED HAEMOLYSIS AND LIPID PEROXIDATION : EFFECT OF VITAMIN E, D AND ANTIOXIDANTS

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### ABSTRACT

Vitamin A-induced haemolysis of rabbit and human erythrocytes is inhibited by Vitamins E, D and ascorbic acid, and by glutathione and cysteine. The A-induced haemolysis is not related to lipid peroxidation of the erythrocyte membranes, nor is it due to glutathione instability.

**I**n *in-vitro* systems, it was demonstrated<sup>1,2</sup> that vitamin A alcohol induced lysis of erythrocytes. Lucy and Dingle<sup>3</sup> had shown that  $\alpha$ -tocopherol acetate, squalene or vitamin K, inhibited haemolysis by vitamin A alcohol and suggested that the inhibition of haemolysis is a feature of compounds having isoprenoid chain. However, these authors also reported<sup>3</sup> on the basis of preliminary experiments, that lipid peroxidation as measured by thiobarbituric acid may be concerned in the lysis of erythrocytes by vitamin A alcohol and that compounds like  $\alpha$ -tocopherol acetate that protect from peroxidation, also prevent haemolysis. This theory is compatible with our earlier observation<sup>4</sup> on the mechanism of dialuric acid-induced haemolysis of erythrocytes from vitamin E deficient rats, which can be prevented by dietary selenium or supplements of vitamin E. It is therefore reasonable to argue that vitamin A-induced haemolysis may also result from peroxidation of the erythrocyte-lipids, especially in the light of the findings of Moore and Sharman<sup>5</sup>, who had shown that erythrocytes from rats, fed excess of vitamin A, were easily haemolysed by dialuric acid as in the case of the erythrocytes of E deficient rats. However, the results presented here demonstrate that vitamin A-induced haemolysis of normal human and rabbit erythrocytes was not related to lipid peroxidation.

### METHODS

Erythrocytes of humans and rabbits from venous blood, freshly withdrawn into equal volume of cold Alsever's solution<sup>6</sup> were sedimented, washed free of plasma twice with saline phosphate buffer (pH 7.4) and resuspended in saline phosphate to give a 1:40 dilution of cells. Vitamin A alcohol, synthetic, from Hoffman L-Roche, purified by alumina chromatography<sup>7</sup> (8% ethanol in petroleum ether fraction being used) in required amounts was taken up in 0.05 ml ethanol and 5 ml measured cell suspensions added for haemolysis studies, the rest of the procedure of incubation and measurement of haemolysis being as described by Dingle and Lucy<sup>1</sup>.

### RESULTS

Both rabbit and human cells were used for the following studies. The results of a mean of 10 different experiments each showed that 8, 30 and 75 micromoles of vitamin A alcohol/ml produced 3%, 75% and 96% haemolysis at the end of 30 min. incubation at 37° C. A time course study indicated that with 35 micromoles of A alcohol/ml there was progressive haemolysis from 20% in 5 min. to 75% in 30 min. In another series of experiments, where aliquots were used for measuring the amounts of lipid peroxidation by thiobarbituric acid<sup>4</sup>, it was observed that while 35 micromoles vitamin A alcohol (30 mins. incubation) produced 80% haemolysis there was no production of the T.B.A. pigment (Table I); even when haemolysis was complete with 100 micromoles of vitamin A/ml no evidence of peroxidation of the erythrocyte lipids was obtained (Table I).

TABLE I

*Lipid peroxidation and vitamin A-induced haemolysis.*

Vitamin A alcohol/ml (Micromoles)	Incubation period (Min.)	Per cent Haemolysis	TBA Values (Klett-readings)
Control (Ethanol)	30	3	10
35	.. Zero time	3	10
	15	40	12
	30	80	13
	60	88	11
7	.. 30	3	10
21	.. 30	15	11
35	.. 30	80	12
70	.. 30	88	12
105	.. 30	100	10

Mean value of 3 different experiments with human cells (1:40) in saline PO<sub>4</sub>-buffer. TBA values given as Klett-Summerson readings using green filter, the procedure essentially according to Krishnamurthy and Bieri<sup>4</sup>.

Table II summarises the results of the effects of ascorbic acid,  $\alpha$ -tocopherol, tocopherol acetate, calciferol, glutathione, and cysteine. Both vitamins E and D inhibited haemolysis, ascorbic acid at low concentrations prevented haemolysis, but at higher

TABLE II  
Influence of additives on the haemolysis of cells  
induced by vitamin A

Factors added/ $\mu$ g/ml	Per cent Haemolysis	Per cent Inhibition of Haemolysis
Control (35 micromoles A alcohol)	88	—
$\alpha$ -Tocopherol		
10 ..	42	53
20 ..	33	63
30 ..	10	88
$\alpha$ -Tocopheryl acetate		
30 ..	15	83
Calciferol		
10 ..	14	84
20 ..	10	88
30 ..	8	91
Ascorbic acid		
50 ..	5	96
100 ..	85	3
200 ..	100	14 (activation)
Cysteine		
25 ..	45	44
50 ..	5	96
Glutathione		
25 ..	30	66
50 ..	5	96
Antioxidants		
Ethoxyquin 100 ..	86	3
DPPD 100 ..	88	Nil
BHT 100 ..	86	3
DAH 100 ..	88	Nil

35  $\mu$  moles A alcohol/ml used for producing haemolysis. Fat soluble factors added in 0.05 ml ethanol along with vitamin A. Incubation at 37° C for 30 min.

Results of mean of 4-different experiments.

levels increased haemolysis. The sulfhydryl compounds, glutathione and cysteine, were also effective in arresting the haemolysis. The stability of glutathione during haemolysis measured as described by Beutler<sup>8</sup>, remained unimpaired; various synthetic antioxidants, santoxin, (Ethoxyquin; 1, 2-dihydro-6-ethoxy-2, 2, 4 trimethyl quinoline), DPPD (N, N', diphenyl P-phenylene diamine), BHT (2, 6-Distert, butyl-p-cresol), DAH (2, 5-Bis 1, 1-dimethyl propyl hydroquinone), obtained through courtesy of Dr. J. G. Beiri, N. I. H. Bethesda, Md, were tried for protection against vitamin A-induced haemolysis and the results were negative (Table II). In separate studies, it was found that the effect of vitamin A in inducing haemolysis is present in the retinol, as experiments with human and rabbit

cells using  $\beta$ -carotene, vitamin A esters, the acetate and the naturally occurring ester<sup>9</sup> the plamitate, did not produce haemolysis. This is in accordance with an earlier report<sup>10</sup> that the cells do not contain the necessary vitamin A esterase for hydrolysis of these esters into the active form, the retinol.

#### DISCUSSION

The results obtained in the present study clearly demonstrate that unlike the oxidative fragility of the vitamin E deficient cells, the haemolysis by retinol is not due to lipid peroxidation of the erythrocyte membrane lipids in contradistinction to the preliminary reports, of Lucy and Dingle<sup>3</sup>.

In fact, in model systems (K. Sree Kumar and S. Krishnamurthy, unpublished observations) using linoleic acid emulsion containing haemoglobin as catalyst, vitamin A alcohol acted as a potent antioxidant confirming the reports of Hunter *et al.*<sup>11/12</sup> that both lysis and lipid peroxidation of the isolated liver mitochondria are prevented by retinol and of Green *et al.*<sup>13</sup> that added vitamin A could prevent *in-vitro* lipid peroxidation in liver hemogenates.

The results with synthetic antioxidants studied here confirm the report of Lucy and Dingle<sup>3</sup> who also found that DPPD did not affect the retinol-induced lysis. Calciferol at low concentrations (10 to 30  $\mu$ g/ml of incubation mixture) markedly inhibited the haemolysis although Lucy and Dingle<sup>3</sup> reported that calciferol inhibited only 5 to 8% of haemolysis at these concentrations and had no effect at higher concentrations. It may be pointed out that we have studied haemolysis for a period of 30 minutes while Lucy and Dingle used 15 minutes period for evaluating the effect of added compounds on haemolysis.

Glutathione, cysteine and ascorbic acid (at low concentrations) also inhibited haemolysis. Vitamin E deficiency in rats is accompanied by impairment of ascorbic acid synthesis and increase in peroxidation in rat liver<sup>14</sup>. In the present study retinol-induced haemolysis was inhibited by low concentration of ascorbate and stimulated by higher concentrations and there was no effect on peroxidation of erythrocyte lipids during haemolysis by any of these concentrations of ascorbate.

The above findings, therefore, may be taken to suggest, *inter alia*, that the haemolysis inhibitory activity is not specifically associated with the long isoprenoid content of the substances, as suggested by Lucy and Dingle<sup>3</sup>. For instance, the requirements of effective inhibition of A-induced haemolysis do not appear to be rigid, because a whole spectrum of unrelated compounds was effective, while synthetic antioxidants were without any

effect. It may be pointed out here that the effect of vitamin A alcohol, in damaging the lysosomal membranes and thereby releasing the lysosomal enzymes, reported by Fell and Dingle<sup>15</sup> as the specific physiological action of vitamin A in controlling membrane structures does not appear to be very specific either in as much as testosterone propionate, a compound structurally unrelated to vitamin A, inhibited bone growth in organ culture experiments and also released acid phosphatase and cathepsins from normal rat liver isolated lysosomes<sup>16</sup>.

It is more possible that the oxidative destruction of vitamin A alcohol itself by red cells as was demonstrated by Pollard and Bieri<sup>17</sup> and Dingle and Lucy<sup>2</sup> initiated the damage of the cellular membranes, thereby resulting in lysis without any co-oxidation of the lipid moieties of the erythrocyte membrane. However, attention is also drawn to the recent hypothesis put forward by Lucy<sup>18</sup> to explain the fusion of biological membranes and suggesting that the lytic action of retinol was due to the presence of a high proportion of globular lipid micelles in membranes challenged by retinol.

We are grateful to Dr. K. Madhavan Kutty, Principal, for encouragement, and Dr. M. S. P. Nair for help in preliminary studies.

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## IMPORTANCE OF O-R POTENTIAL IN INITIATING CULTURES OF AXENICALLY GROWN *ENTAMOEBA HISTOLYTICA* FROM SMALL INOCULA\*

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### ABSTRACT

It has been shown that maintenance of suitable negative O-R potential during sub-culturing axenically grown *Entamoeba histolytica* plays a very important role in initiating cultures from small inocula. An inoculum of 250 amoebae/ml of Diamond's TP-S-1-medium containing 0.3% cysteine without ascorbic acid plus 0.05% agar, given carefully at the bottom of screw-capped tubes and incubating the tubes in upright position without inverting them, yielded 200,000 amoebae/ml of the medium in 15 days. The time taken for the amoebae to reach the maximum population depended on the size of the inoculum.

IT is well known that maintenance of strict anaerobic condition is necessary for the growth of *Entamoeba histolytica* in association with bacteria (Chang<sup>1</sup>). In spite of this finding, no attention has been paid to study the effect of

O-R potential on the growth of axenic *E. histolytica*. Diamond<sup>2</sup> in 1961 used 0.1% L-cysteine HCl and 0.02% l-ascorbic acid in a diphasic medium to grow *E. histolytica* (strain 200 : NIH) axenically. The presence of 0.05% agar in the overlay was found to be necessary for the growth of amoebae. In a well-established culture, an inoculum of 50,000 amoebae

\* Communication No. 1828 from Central Drug Research Institute, Lucknow,