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FREE RADICAL CHAIN OXIDATION OF ERYTHROCYTE GHOST MEMBRANES BY OXYGEN

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The oxidation of human and rat erythrocyte ghost membranes has been performed at 37 °C in the presence of radical initiator. It was found that erythrocyte membranes are oxidized by free radical chain mechanism by oxygen with long kinetic chain length of 10 to 100. This is the first clear evidence that shows the radical chain oxidation of biological membranes.

The non-enzymatic oxidation of lipids in tissue *in vivo* and *in vitro* has received much attention recently in connection with its pathological effects and aging.[1] The erythrocytes contain polyunsaturated fatty acid esters which are quite susceptible to oxidation. The oxidation of erythrocytes by hydrogen peroxide, xanthine oxidase, and organic hydroperoxides has been known to induce hemolysis and membrane damage.[2]

On the other hand, the oxidation of erythrocytes by molecular oxygen has not been studied in detail. One important question is whether or not the biological membranes are oxidized by free radical chain mechanism and, if so, how long the chain lasts. It has been found that the artificial liposomal membranes are oxidized by free radical chain mechanism and that the kinetic chain length is considerably long.[3,4] We wish to present here the experimental evidence which shows, for the first time, that the erythrocyte ghost membranes are oxidized by molecular oxygen by free radical chain mechanism with long kinetic chain length.

Healthy human blood was obtained from Central Blood Center, Japanese Red Cross. Rats fed by vitamin E-deficient and control diets for 10-12 weeks were gifted. Human blood and heparinized rat blood were centrifuged and the plasma and buffy coats were removed. The cells were washed three times with cold 0.9% aqueous NaCl. Ghosts were prepared by the method of either Dodge et al.[5] or Ingold et al.[6] About 97% of hemoglobin was removed by the former method and 100% by the latter. Ghosts prepared by the Dodge method were treated with carbon monoxide gas to convert oxyhemoglobin to carboxyhemoglobin.

Appropriate amounts of ghost suspension in 0.9% aqueous NaCl and an isotonic solution (pH 7.4) containing phosphate, NaCl, and water-soluble radical initiator were taken into a reaction vessel. 2,2'-Azobis(2-amidinopropane) hydrochloride (AAPH) was used as a radical initiator. Reaction vessel was immersed in a water bath kept at 37 °C to start the oxidation of ghosts. Oxidation of ghosts was followed by the decrease of oxygen concentration in the reaction solution with oxygen electrode.

Figure 1 shows the results of oxidation of erythrocyte membranes initiated with AAPH. In the absence of AAPH, little oxygen uptake was observed. On the other hand, when AAPH was added and the initiating radicals were generated at a constant rate in the aqueous phase, a constant rate of oxygen uptake was observed.

Under these circumstances, the oxidation of lipid (LH) of erythrocyte membranes proceeds by the following mechanism.[3,4]

Initiation:
$$\text{A-N=N-A} \longrightarrow [\text{A}^{\cdot} \quad \text{N}_2 \quad {}^{\cdot}\text{A}] \xrightarrow{\text{O}_2} 2e\text{AO}_2^{\cdot} + e\text{N}_2 \qquad (1)$$
$$\xrightarrow{\text{O}_2} (1-e)\text{A-A} + (1-e)\text{N}_2 \qquad (2)$$
$$\text{AO}_2^{\cdot} + \text{LH} \xrightarrow{\text{O}_2} \text{AOOH} + \text{LO}_2^{\cdot} \qquad (3)$$

Propagation:
$$\text{LO}_2^{\cdot} + \text{LH} \longrightarrow \text{LOOH} + \text{L}^{\cdot} \qquad (4)$$
$$\text{L}^{\cdot} + \text{O}_2 \longrightarrow \text{LO}_2^{\cdot} \qquad (5)$$

Termination:
$$2\text{LO}_2^{\cdot} \longrightarrow \text{non-radical products} \qquad (6)$$

In this scheme, A-N=N-A is an azo initiator, e is the efficiency of radical production, k_d is the rate constant for unimolecular decomposition of initiator, and k_p and k_t are the rate constants for reactions 4 and 6, respectively. The rate of oxidation is given by Eq. 7, where R_p and R_i are the rate of propagation and initiation, respectively. R_i is expressed by Eq. 8. R_i can be calculated by Eq. 9,[3,7] where t_{inh} is the induction period produced in the presence of antioxidant (IH) and n is the stoichiometric number of peroxy radicals trapped by each antioxidant.

$$R_p = -d[\text{O}_2]/dt = R_i^{\frac{1}{2}}[\text{LH}]k_p/(2k_t)^{\frac{1}{2}} \qquad (7)$$

$$R_i = 2ek_d[\text{A-N=N-A}] \qquad (8) \qquad R_i = n[\text{IH}]/t_{inh} \qquad (9)$$

As shown in Fig. 1, the induction period was observed in the oxidation of human erythrocyte ghosts. The results obtained for some human and rat erythrocyte ghosts are summarized in Table 1. The rate of initiation was calculated from the induction period and the amount of α-tocopherol measured in the membranes. Vitamin E is the only chain breaking antioxidant in erythrocyte membranes[8] and α-tocopherol was predominant tocopherol in ghosts. Furthermore, α-tocopherol disappeared at the end of induction period. The number n for α-tocopherol was taken as 2.[3,7] As shown in Table 1, the average value for ek_d was obtained as 1.64×10^{-7} s^{-1}. This value is in good agreement with $ek_d = 1.53 \times 10^{-7}$ s^{-1} obtained for AAPH in soybean phosphatidylcholine liposome system at 37 °C (unpublished data).

Some pertinent results of the oxidations of erythrocyte ghost membranes of human and vitamin E-deficient and control rats are shown in Table 2. It was found that the rate of oxidation was proportional to the square root of initiator concentration (Runs 1, 2, 7, and 8 in Table 2) as expected from Eq. 7, suggesting that the chains are terminated by statistical bimolecular interaction of peroxy radicals. Table 2 shows that erythrocyte ghosts are oxidized with long kinetic chain length of 10 to 100. Similar results were obtained when rate of oxygen uptake was measured by a pressure transducer.[4]

The results given above clearly show that the erythrocyte membranes are

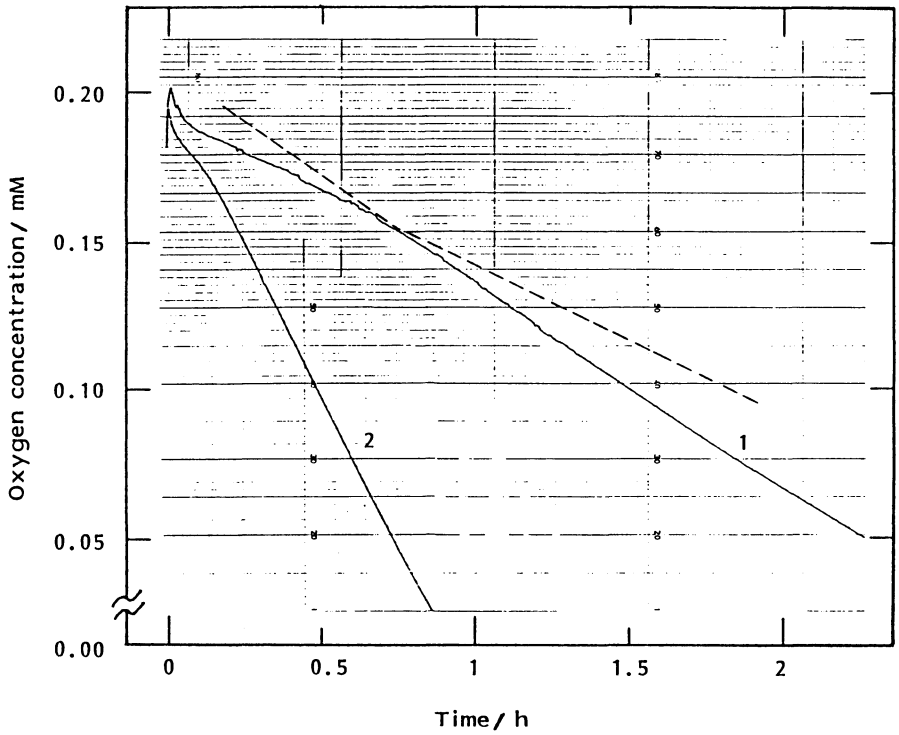

Fig. 1. AAPH-initiated oxidation of human erythrocyte ghost membranes prepared by the method of Dodge et al.[5] in 10 mM phosphate buffer (pH 7.4) at 37 °C. See Runs 1 (line 1) and 8 (line 2) in Table 2.

oxidized by free radical chain mechanism, when the initial radicals are formed in aqueous phase. The kinetic chains must be longer in biological systems than those obtained in this in vitro study, since the kinetic chain length is inversely proportional to the half-power of the rate of chain initiation and since the rate of radical production in vivo must be smaller than that in the present study.

Table 1. Induction period observed in the oxidation of human and rat erythrocyte ghost membranes in aqueous suspension initiated with AAPH at 37 °C

Run No.	1	2	3	4	5	6
Erythrocyte	Man	Man	Man	Man	Man	Rat
Ghost preparation[a]	A	A	B	B	B	A
Fatty acids, mg/l suspension	192	192	169	75.6	233	245
α-Tocopherol, μmol/l suspension	0.488	0.488	0.348	0.413	1.27	1.20
AAPH, mmol/l suspension	0.623	1.25	1.25	0.498	1.69	1.25
t_{inh}, s	4600	2660	2160	4500	4500	5040
$10^7\ ek_d$, s^{-1}	1.70	1.47	1.29	1.84	1.67	1.90

a) A: Method of Dodge et al.,[5] B: method of Ingold et al.[6]

Table 2. Oxidation of erythrocyte ghost membranes in aqueous suspension initiated with AAPH at 37 °C

Run No.	Erythrocyte[a]	Ghost[b]	Fatty acids[c]	Protein[d]	AAPH[e]	R_i[f]	R_p[g]	kcl[h]
1	Man	A	192	1.22	0.623	0.199	14.4	72.4
2	Man	A	192	1.22	1.25	0.399	18.8	47.1
7	Man	A	192	1.22	2.49	0.795	24.9	31.3
8	Man	A	192	1.22	9.96	3.18	50.4	15.8
9	Man	B	169	0.33	9.96	3.18	35.3	11.1
3	Man	B	169	0.33	1.25	0.399	11.4	28.6
10	E.R	A	266	-	0.627	0.200	19.9	99.5
6	E.R	A	266	-	1.25	0.399	21.2	53.1
11	C.R	A	245	-	1.25	0.399	16.0	40.1
12	E.R	B	142	0.48	1.25	0.399	15.5	38.8
13	C.R	B	151	0.34	1.25	0.399	11.5	28.8

a) E.R: vitamin E-deficient rat; C.R: control rat. b) A, B: ghosts prepared by the method of Dodge et al.[5] or Ingold et al.,[6] respectively. c) In mg/l suspension. d) In g/l suspension. e) In mmol/l suspension. f) Rate of chain initiation in nmol radcial/l suspension/s, $R_i = 2 \times 1.6 \times 10^{-7}$[AAPH]. g) Rate of propagation in nmol O_2/l suspension/s. h) Kinetic chain length = R_p/R_i.

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