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Oxidation of biological membranes and its inhibition. Free radical chain oxidation of erythrocyte ghost membranes by oxygen

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The oxidation of human and rat erythrocyte ghost membranes by molecular oxygen has been performed in an aqueous suspension at 37°C. A constant rate of oxygen uptake was observed in the presence of radical initiator. α-Tocopherol in the membrane suppressed the oxidation and the induction period was clearly observed. α-Tocopherol decreased linearly during the induction period and when it was depleted the induction period was over and a rapid oxidation started. The rate of oxidation was proportional to the square root of the rate of initial radical generation. The kinetic chain length, the ratio of the rate of propagation to that of initiation, was long, ranging from 7 to 100. These results indicate that the erythrocyte ghost membranes are oxidized by a free radical chain mechanism by molecular oxygen. Among the fatty acids of membrane lipids, polyunsaturated fatty acids were oxidized exclusively. Proteins as well as polyunsaturated fatty acids were oxidized and the formation of the high- and low-molecular-weight proteins and the decrease of protein bands were observed on gel electrophoresis.

Introduction

Erythrocytes are very susceptible to peroxidation [1]: The erythrocyte membrane is rich in polyunsaturated fatty acids, erythrocytes are continuously exposed to high concentration of oxygen and contain hemoglobin which may catalyze the oxidiation. Recently the non-enzymatic oxidation of lipids in tissue in vivo and in vitro has received much attention in connection with its pathological effects and aging [2,3]. The oxidation of erythrocyte membranes serves as a good model to study the peroxidative damage to biological membranes.

The oxidations of erythrocytes by hydrogen peroxide, xanthine oxidase, and organic hydroperoxides have been known to induce hemolysis and membrane damage [4–19]. Photooxidation of human erythrocytes catalyzed by copper ion has been also reported recently [20]. 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 a free radical chain mechanism and, if so, how low is that chain. It has been found that artificial liposomal membranes are oxidized by a free radical chain mechanism and that the kinetic chain length is long [21,22]. Another question is which component of the membrane is oxidized preferentially. We wish to present here the experimental evidence

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Abbreviation: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride.

which shows, for the first time, that the erythrocyte ghost membranes are oxidized by molecular oxygen by a free radical chain mechanism with long kinetic chain length and that proteins as well as polyunsaturated fatty acids of membrane lipids are oxidized considerably to give the high-molecular-weight and the low-molecular-weight compounds.

Methods

Materials. Healthy human blood was obtained from Central Blood Center, Japanese Red Cross. Sodium citrate was used as an anticoagulant. Rats fed by vitamin E-deficient and control diets for 10–12 weeks were given by Drs. Mino, Nakagawa, Tamai, and Miki, Osaka Medical College. 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. [23], or Burton et al. [24], and called the pink ghosts and the white ghosts, respectively. About 98% of hemoglobin was removed by the former method and 100% by the latter. Ghosts were stored as suspension in 0.9% aqueous NaCl. Ghosts prepared by the Dodge method were treated with carbon monoxide gas before the oxidation to convert oxyhemoglobin to carboxyhemoglobin. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was used as a water-soluble radical initiator in order to generate radicals at known and constant rate in an aqueous region. This is essential for quantitative kinetic studies.

Analytical methods. Tocopherols in ghost mem-

TABLE I

METHOD OF PREPARATION AND CHEMICAL ANALYSIS OF GHOSTS

Ghost	I	II	III	IV	V	VI
Erythrocyte source [a]	Man	Man	Man	Man	E.R.	C.R.
Preparation [b]	A	A	B	B	B	B
Protein [c]	4.86	3.20	1.32	1.04	1.91	1.37
α-Tocopherol [d]	1.95	1.70	1.39	1.65	< 0.05	2.74
Fatty acid [e]	768	358	677	302	567	604
Fatty acid composition (in mole %)						
14:0	1.5	tr	1.4	tr	1.4	1.3
16:0	29.8	24.9	25.9	23.4	25.9	26.1
16:1	0.5	tr	0.4	tr	0.5	0.4
18:0	13.6	16.6	14.0	15.8	13.0	13.4
18:1	13.1	18.1	12.6	16.2	9.5	8.9
18:2	8.8	14.2	15.6	11.7	24.4	22.1
20:3	1.0	tr	0.8	tr	0.2	0.4
20:4	13.6	12.3	15.0	11.0	21.3	23.3
22:0	2.3	1.6	1.7	3.0	0.4	0.5
22:4	1.4	1.1	1.4	1.5	1.4	1.5
22:5 + 24:0	4.2	1.3	3.3	3.0	0.7	0.8
22:6 + 24:1	10.2	9.9	7.9	14.4	1.3	1.3
Average molecular weight [f]	289	288	288	283	282	283
Active H [g]	1.79	1.64	1.86	1.82	1.95	2.04

[a] E.R.: vitamin E-deficient rat; C.R.: control rat.
[b] A, B: ghosts prepared by the method of Dodge et al. [23] or Burton et al. [24], respectively.
[c] In g/l suspension measured by the method of Lowry et al. [26].
[d] In μmol/l suspension measured by the method of Abe et al. [25].
[e] In mg/l suspension determined as described in the experimental section.
[f] Molar ratio of 22:5/24:0 and 22:6/24:1 was assumed to be 1 [35].
[g] Average number of bisallylic hydrogens in one molecule of fatty acid calculated from the fatty acid composition. For example, linoleic and arachidonic acids have 2 and 6 bisallylic hydrogens, respectively.

branes were estimated by the method of Abe et al. [25] using high pressure liquid chromatography. Finepak SIL-CN (10 μm, 4.6 mm (diameter) $\times$ 250 mm), Japan Spectroscopic Co., and hexane/isopropyl alcohol/acetic acid (1000 : 10: 1, v/v/v) were used as a column and an eluent, respectively. Proteins were determined by the method of Lowry et al. [26]. Membrane lipids were extracted by the method of Rose et al. [27] and methyl eicosanoate was used as an internal standard. The amounts and composition of fatty acid of membrane lipids were determined by GLC using Shinchrome E-71, Shimadzu Seisakusho, as a column after the treatment with HCl/methanol or tetramethylammonium hydroxide/methanol to obtain free acid methyl esters. Table I summarizes the method of preparation and chemical properties of ghosts. SDS-polyacrylamide gel electrophoresis was performed with 5% and 10% gels for condensation and separation, respectively. Two parts of sample suspension were mixed with one part of an aqueous solution containing 0.15 M Tris-HCl (pH 6.8), 3% SDS, 30% glycerol, 3% β-mercaptoethanol, and 0.03% Bromophenol blue. The amount of protein layered was 31–38 μg.

Oxidation procedures. Appropriate amounts of ghosts suspension and an isotonic solution (pH 7.4) containing phosphate (10 mM in final reaction solution), NaCl, and AAPH were taken into a reaction vessel. The reaction vessel was immersed in a water bath kept at 37°C to start the oxidation of ghosts. The rate of oxidation of ghosts was followed by the decrease of oxygen concentration in the reaction solution with Biological Oxygen Monitor, Model YSI 53 (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) or by the pressure transducer as previously reported [22].

Results and Discussion

Fig. 1 shows the results of oxidation of the white ghosts prepared by the method of Burton et al. [24] 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. Similar results were observed for the pink ghosts prepared by the method of Dodge et al. [23].

Under these circumstances, the oxidation of lipid (LH) of erythrocyte membranes proceeds by the following mechanism [21,22].

$$A\text{-}N{=}N\text{-}A \xrightarrow{k_d} [A\cdot N_2\cdot A] \xrightarrow{O_2} 2eAO_2^{\cdot} + eN_2 \quad (1)$$

$$\rightarrow (1-e)A\text{-}A + (1-e)N_2 \quad (2)$$

$$AO_2^{\cdot} + LH \xrightarrow{O_2} AOOH + LO_2^{\cdot} \quad (3)$$

Propagation:

$$\rightarrow LO_2^{\cdot} + LH \xrightarrow{k_p} LOOH + L^{\cdot} \quad (4)$$

$$L^{\cdot} + O_2 \rightarrow LO_2^{\cdot} \quad (5)$$

Termination:

$$2LO_2^{\cdot} \xrightarrow{k_t} \text{non-radical products} \quad (6)$$

In this scheme, A-N=N-A is an azo initiator ($A{=}HCl\cdot NH{=}C(NH_2)\text{-}C(CH_3)_2\text{-}$), e is the efficiency of free radical production, k_d is the rate constant for unimolecular decomposition of an initiator, and k_p and k_t are the rate constants for reactions 4 and 6, respectively. The rate of oxidation is given by Eqn. 7 [21,22]:

$$R_p = -d[O_2]/dt = R_i^{1/2}[LH]k_p/(2k_t)^{1/2} \quad (7)$$

where R_p and R_i are the rate of chain propagation and chain initiation, respectively. R_i is expressed by Eqn. 8.

$$R_i = 2ek_d[A\text{-}N{=}N\text{-}A] \quad (8)$$

The rate of initiation can be calculated from Eqn. 9 by measuring the induction period (t_{inh}) produced by the addition of antioxidant (IH) [21,22]:

$$R_i = n[IH]/t_{inh} \quad (9)$$

where n is the stoichiometric number of peroxy radicals trapped by each antioxidant.

Fig. 1 shows that the induction period was

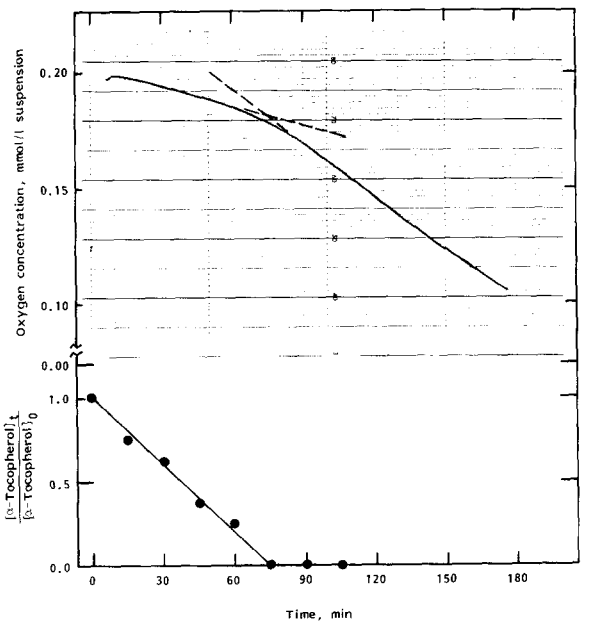


Fig. 1. Oxygen uptake (top) and the decrease of α-tocopherol (bottom) in the oxidation of human erythrocyte ghosts initiated with AAPH in 10 mM phosphate buffer (pH 7.4) at 37°C. See Run 5 in Table II.

observed in the oxidation of human erythrocyte ghosts initiated with AAPH. Fig. 1 also shows that α-tocopherol decreased linearly with time during the induction period and disappeared at the end of the induction period. Vitamin E is the only chain breaking, oil-soluble antioxidant in erythrocyte membranes [28] and α-tocopherol was a predominant tocopherol in ghosts. The rate of chain initiation was calculated from the induction period and the amount of α-tocopherol observed in the membranes. The number n for α-tocopherol was taken as 2 [21,29] and the results are summarized in Table II. The average value for ek_d was obtained as $1.64 \cdot 10^{-7}$ s^{-1}. This value is in good agreement with $ek_d = 1.53 \cdot 10^{-7}$ s^{-1} obtained for AAPH in soybean phosphatidylcholine liposome system at 37°C [30].

Some pertinent results of the oxidations of erythrocyte ghost membranes of human and vitamin E-deficient and control rats are shown in Table III. Similar results were obtained when the rate of oxidation was measured by a pressure transducer [22]. The data in Table III show that the rate of oxidation was proportional to the square root of initiator concentration as expected from Eqn. 7, suggesting that the chains are terminated by statistical bimolecular interaction of peroxy radicals. The kinetic chain length is defined as the ratio of rate of oxygen uptake to that of radical formation, R_p/R_i. Table III shows that erythrocyte ghosts are oxidized with long kinetic chain length ranging from 7 to 100.

The kinetic chain length longer than 1 observed in this study clearly shows that the erythrocyte ghost membranes are oxidized by a free radical chain mechanism when the initial radical are formed in an aqueous phase. In other words, when one radical generated in an aqueous phase attacks membrane, 7 to 100 molecules of fatty acids (and protein, see later text) are oxidized. The kinetic

TABLE II

INDUCTION PERIOD OBSERVED IN THE OXIDATION OF HUMAN AND RAT ERYTHROCYTE GHOST MEMBRANES IN AN AQUEOUS SUSPENSION INITIATED WITH AAPH AT 37°C

Run No.	1	2	3	4	5	6
Ghost [a]	I	I	III	IV	IV	[b]
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 [c], s^{-1}	1.70	1.47	1.29	1.84	1.67	1.90

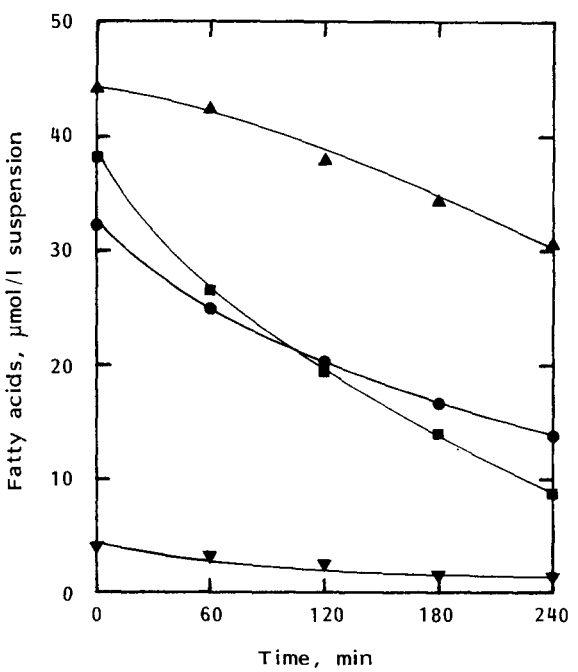
[a] See Table I for definition.
[b] Prepared by the method of Dodge et al. [23] from control rat.
[c] Calculated from Eqns. 8 and 9, $ek_d = [\alpha\text{-Tocopherol}]/(t_{inh} \cdot [AAPH])$.

TABLE III

OXIDATION OF ERYTHROCYTE GHOST MEMBRANES IN AN AQUEOUS SUSPENSION INITIATED WITH AAPH AT 37°C

Run No.	Ghost [a]	Fatty acid [b]	Protein [c]	AAPH [d]	R_i [e]	R_p [f]	R_p/R_i [g]
1	I	192	1.22	0.623	0.199	14.4	72.4
2	I	192	1.22	1.25	0.399	18.8	47.1
7	I	192	1.22	2.49	0.795	24.9	31.3
8	I	192	1.22	9.96	3.18	50.4	15.8
9	I	192	1.22	10.1	3.22	49.2	15.3
10	II	89.5	0.80	9.96	3.18	33.1	10.4
11	II	89.5	0.80	9.96	3.18	32.2	10.1
12	III	169	0.33	9.96	3.18	35.3	11.1
13	III	169	0.33	10.1	3.22	31.4	9.8
14	IV	75.6	0.26	10.1	3.22	23.3	7.2
15	IV	75.6	0.26	10.1	3.22	27.8	8.6
16	V	142	0.48	9.96	3.18	55.4	17.4
17	VI	151	0.34	9.96	3.18	40.1	12.6
18	[h]	266	[j]	0.627	0.200	19.9	99.5
19	[i]	245	[j]	0.627	0.200	13.6	68.0

[a] See Table I for definition.
[b] In mg/l suspension.
[c] In g/l suspension.
[d] In mmol/l suspension.
[e] Rate of chain initiation in nmol radical/liter suspension per s, $R_i = 3.19 \cdot 10^{-7}$ [AAPH].
[f] Rate of propagation in nmol O_2/liter suspension per s.
[g] Kinetic chain length.
[h,i] Prepared by the method of Dodge et al. [23] from vitamin E-deficient and control rats, respectively.
[j] Not determined.



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 biological systems must be smaller than that in the present study.

Fig. 2 shows the decrease in fatty acids of membrane lipids during the oxidation of the pink ghosts in an aqueous suspension initiated with AAPH at 37°C. Polyunsaturated fatty acids decreased with time and arachidonic acid was consumed faster than linoleic acid. This must be ascribed to the higher number of active hydrogens in arachidonic acid than that in linoleic acid. We have previously observed that the oxidizabilities of

Fig. 2. The decrease of polyunsaturated fatty acids of membrane lipids in the oxidation of human erythrocyte ghosts initiated with AAPH in 10 mM phosphate buffer (pH 7.4) at 37°C. See Run 11 in Table III. 18:2 (▲), 20:4 (■), 22:5(+24:0) (▼), 22:6(+24:1) (●).

bisallylic hydrogens in different polyunsatured fatty acids are similar independent of parent polyunsaturated fatty acids and reaction medium [22]. Therefore, the relative susceptibility of polyunsaturated fatty acids to oxidation is determined by the number of active hydrogens and concentration.

Fig. 3 shows the plot of Eqn. 7 for the oxidation of human erythrocyte ghosts. Polyunsaturated fatty acids are the major substrate in the oxidation of ghosts (see later discussion), and the rate of oxidation was expressed in mol O_2/liter fatty acid per s. The rate of initiation was expressed similarly in mol radical/liter fatty acid per s. The concentration of fatty acid was calculated from its assumed density (0.9 g/ml fatty acid) and the average molecular weight of fatty acid (287 g/mol) as 900/287 = 3.14 mol/liter fatty acid (see Table I). The slope represents the ratio of the rate constants, $k_p/(2k_t)^{1/2}$, which is often referred to as the oxidizability of the substrate [21,22]. The oxidizabilities of fatty acid per active hydrogen (see Table I) were 0.0098 and 0.0071 (s · mol/liter fatty acid/active H)$^{-\frac{1}{2}}$ for the ghosts prepared by the method of Dodge et al. [23] and Burton et al. [24], respectively. These numbers are smaller than

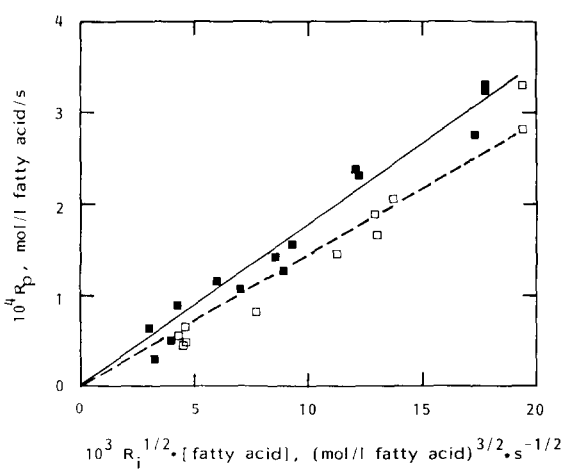


Fig. 3. Plot of the rate of oxidation (R_p) against $R_i^{1/2}$·[fatty acid] in the AAPH-initiated oxidation of human erythrocyte ghosts prepared by the method of Dodge et al. (■) and Burton et al. (□) in 10 mM phosphate buffer (pH 7.4) at 37°C under air. Solid and dotted lines show the average slopes for the ghosts prepared by the Dodge, and Burton and Ingold methods, respectively.

the number (0.03) observed in the oxidation of soybean phosphatidylcholine unilamellar liposome at 37°C under air [30].

Table IV summarizes the ratio of the amount of oxygen uptake to that of polyunsaturated fatty acids decreased in the oxidation of human erythrocyte ghosts. The ratio was larger than 3 and increased with reaction time: for example, when $11.6 \cdot 10^{-6}$ mol/l of initiating radicals were generated in the aqueous phase in 60 min ($3.19 \cdot 10^{-7} \cdot 0.0101 \cdot 3600$), $177 \cdot 10^{-6}$ mol/l of oxygen were taken up and $40.8 \cdot 10^{-6}$ mol/l of polyunsaturated fatty acids were oxidized (see Run 8 in Table III and first entry in Table IV). The ratios observed previously were almost 1.0 for the oxidations of methyl linoleate in homogeneous solution [31] and in a water dispersion [32], and those of dilinoleoylphosphatidylcholine in solution and as liposome [22], 1–2 for the oxidation of methyl linolenate in solution [31] and soybean and egg yolk phosphatidylcholines as liposome [22], and 2–2.5 for the oxidation of rat-liver phosphatidylcholine as liposome [22]. Comparing the above numbers and the composition of polyunsaturated fatty acids in the ghosts and lipids, polyunsaturated fatty acids are the major substrate in the oxidation of ghosts. But some other substrates must be also oxidized in the oxidation of erythrocyte ghosts. As shown in Table IV, the rate of oxidation of ghosts I was faster than that of ghosts III when they were oxidized under similar conditions. Fig. 3 shows that the pink ghosts are oxidized faster than the white ghosts at constant fatty acid concentration. These results imply that the rate of oxidation increases with an increase of proteins, suggesting not only polyunsaturated fatty acid but also proteins were oxidized appreciably.

Figs. 4 and 5 show the SDS-polyacrylamide gel electrophoresis of unoxidized and oxidized erythrocyte ghosts prepared by the method of Dodge et al. [23] and Burton et al. [24], respectively. The pink ghosts prepared by the Dodge method lack band 6 and the white ghosts prepared by the Burton and Ingold method lack bands 3, 5, and 6. This indicates that the pink ghosts preserve the structure of erythrocyte membranes better than the white ghosts, but the white ghosts are free from hemoglobin. In the oxidatioan of the pink ghosts, spectrin (bands 1 and 2), the anion channel

TABLE IV

THE RATIO OF THE AMOUNT OF OXYGEN UPTAKE TO THAT OF POLYUNSATURATED FATTY ACIDS (PUFA) DECREASED IN THE OXIDATION OF HUMAN ERYTHROCYTE GHOSTS INITIATED WITH AAPH AT 37°C

Ghost [a]	I	I	II	II	III	III
Protein, g/l suspension	1.22	1.22	0.80	0.80	0.33	0.33
Fatty acid, μmol/l suspension	665	665	311	311	588	588
PUFA, μmol/l suspension	213	213	103	103	226	226
AAPH, mmol/l suspension	10.1	10.1	10.0	10.0	9.96	9.96
Time, min	60	120	60	120	60	120
ΔO_2, μmol/l suspension	177	354	116	232	127	254
ΔPUFA, μmol/l suspension	40.8	68.5	21.8	38.5	41.2	64.9
$\Delta O_2/\Delta$PUFA	4.3	5.2	5.3	6.0	3.1	3.9

[a] See Table I for definition.

(band 3), and the bands between the bands 2 and 3 decreased with the extent of oxidation. On the contrary, the high-molecular-weight protein shown at the top of the gel and the upside of band 1 increased with time. The formation of the low-molecular-weight protein shown between band 3 and the front of electrophoresis was observed at 60 and 90 min oxidiaiton. In the oxidation of the white ghosts, all the bands decreased with time and the high-molecular-weight protein shown at

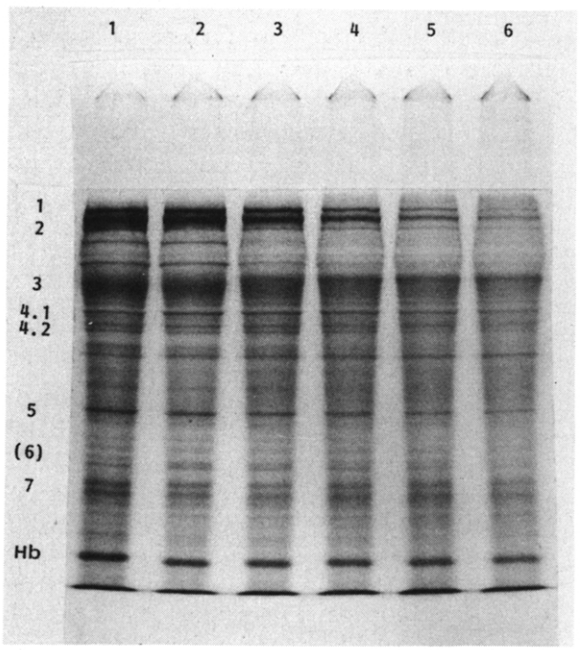


Fig. 4. SDS-polyacrylamide gel electrophoresis of erythrocyte ghosts prepared by the method of Dodge et al. 1: Initial. 2–6: Oxidized with 0.01 M AAPH at 37°C for 0, 30, 60, 90, 120 min, respectively. The concentration of fatty acids of membrane lipids and protein were 322 mg and 1.98 g per liter suspension, respectively. Rate of oxidation was 57.0 nmol O_2/liter suspension per s. The amount of protein layered was 38 μg.

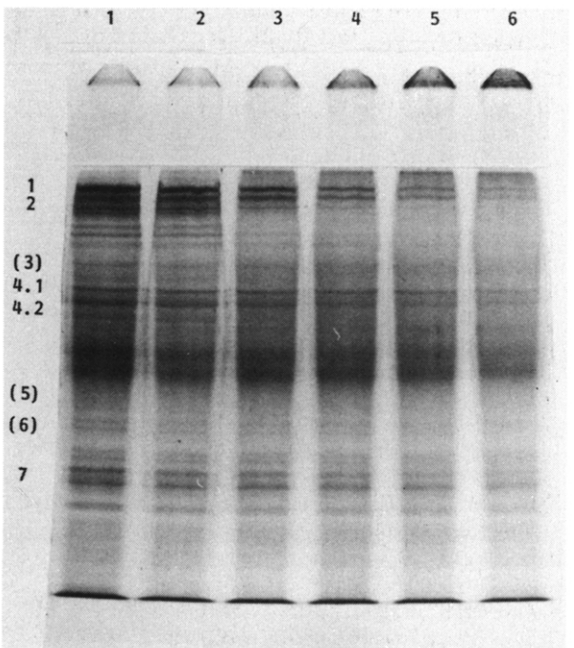


Fig. 5. SDS-polyacrylamide gel electrophoresis of erythrocyte ghosts prepared by the method of Burton et al. 1: Initial. 2–6: Oxidized with 0.01 M AAPH at 37°C for 0, 30, 60, 90, 120 min, respectively. The concentration of fatty acids of membrane lipids and protein were 484 mg and 0.943 g per liter suspension, respectively. Rate of oxidation was 43.9 nmol O_2/liter suspension per s. The amount of protein layered was 38 μg.

the top of the gel and the upside of band 1 increased with time. The low-molecular-weight protein was detectable at 30–90 min oxidation behind the front of electrophoresis. The formation of the high-molecular-weight product has been observed when ghosts were incubated with organic hydroperoxides such as *tert*-butyl hydroperoxide and cumyl hydroperoxide [18] and when albumin was incubated with linoleic acid hydroperoxide [33] and oxidized lipid [34]. Therefore, the high-molecular-weight product observed in the present study must be formed similarly by the interaction of protein and oxidized lipid. On the other hand, the formation of the low-molecular-weight protein must be ascribed to the oxidative degradation of protein.

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