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# VITAMIN E AND THE PEROXIDIZABILITY OF ERYTHROCYTE MEMBRANES IN NEONATES

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We showed the increased susceptibility of neonatal biomembranes to oxidation by a kinetic analysis using an azo compound as a free-radical initiator and red blood cell (RBC) ghosts as a model membrane. When the RBC ghosts were oxidized, oxygen consumption was suppressed during the induction period in which membrane tocopherol was consumed at a constant rate, while increased oxygen uptake was observed after the tocopherol was exhausted. The total tocopherol content was similar in cord, maternal, and adult RBC ghosts, and there were no differences in the induction period ($t_{inh}$) among the three types of ghosts. While the oxygen uptake rate during the induction period ($R_{inh}$) was similar in cord and adult ghosts, the rate in the subsequent phase ($R_p$) was considerably faster in the cord ghosts. Fatty acid analysis in the membrane lipids showed that the active bisallylic hydrogen (active H) content was greater in cord ghosts than in adult ghosts. The active H content closely correlated with the $R_p$, but did not with the $R_{inh}$. The kinetic chain length (KCL), i.e., the ratio of the rate of propagation to that of initiation, was calculated from $R_p$ and tocopherol consumption rate and KCL values were higher in cord ghosts than in adult ghosts. The faster $R_p$ and the higher KCL of the cord ghosts were attributable to a greater active H content rather than to the tocopherol content.

KEY WORDS: Red blood cell ghosts, neonates, polyunsaturated fatty acids, active bisallylic hydrogen atoms, α-tocopherol, azo compounds, oxygen uptake, induction period, kinetic chain length, peroxidizability, hemolysis.

ABBREVIATIONS: AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; Active H, active bisallylic hydrogen; KCL, kinetic chain length: PUFA, polyunsaturated fatty acid; RBC, red blood cell; $R_{inh}$, oxygen uptake rate during the induction period; $R_p$, oxygen uptake rate after the induction period; $t_{inh}$, length of the induction period.

## INTRODUCTION

Oxidation of biomembrane constituents appears to be a deleterious process and has recently been accepted to be involved in a variety of pathological events derived from oxygen toxicity.[1,2] Free radicals attacking biomembranes can produce the oxidative destruction of the polyunsaturated fatty acids in these membranes, which is the well-documented process termed lipid peroxidation.[3] Aerobic organisms are protected against oxygen toxicity by an array of defense mechanisms. Vitamin E (α-tocopherol) is now accepted to function as a potent chain-breaking antioxidant, particularly in biomembranes, by scavenging the chain-carrying peroxyl radicals.[4,5]

It has been previously supposed that newborn infants (especially premature infants) are in a marginally vitamin E-deficient state, due to their low plasma vitamin E levels

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and the increased susceptibility of their RBC to oxidative stress induced by hydrogen peroxide or dialuric acid.[6-9] On the other hand, Martinetz *et al.*[10] and Desai *et al.*[11] have reported that neonates may not be deficient in vitamin E, because the ratio of α-tocopherol to lipid in neonatal plasma is above the critical level for vitamin E deficiency. This ratio has been proposed by Horwitt *et al.*[12] as a very reliable index of vitamin E status. In fact, we found previously that RBC tocopherol levels in premature infants were similar to those in adults despite their increased susceptibility to oxidative stress.[13,14]

In the present paper, we report the results of kinetic studies on oxidation of RBC ghosts using an azo compound as a radical initiator. Azo compounds enable us to generate peroxyl radicals by thermal decomposition at a constant rate and are suitable for the kinetic study.[15,16] We attempt to clarify the reason for the increased susceptibility of cord RBC ghosts to oxidation comparing with adult RBC ghosts. We also examine the peroxidizability of RBC ghosts from pregnant women as an example of hyperlipidemic individuals, who may be more susceptible to oxidative stress than healthy adults.[14,17]

## EXPERIMENTAL PROCEDURES

### *Blood*

Heparinized blood was drawn from healthy pregnant women (21–31 years old) immediately after delivery, and cord blood was simultaneously obtained through the umbilical vein from separated placenta. The gestational age and birth weight of the newborns was 38 ± 2 weeks and 2,815 ± 250 g, respectively. Only healthy infants with Apgar scores of greater than 9 at 5 min after a normal vaginal delivery were selected. All the mothers had no complications during pregnancy and delivery. In addition, healthy male laboratory personal (25–35 years old) were also analyzed. The study protocol was approved by the ethics committee of the college hospital, and the studies were performed after informed consent was obtained from all the subjects.

### *Preparation of RBC Ghosts*

After heparinized blood was centrifuged at 1,000 g for 10 min, the RBC were separated from the plasma and buffy coat, and then washed three times with 10 vol. of 0.15 M NaCl. Ghost membranes were prepared from the RBC by Burton's method.[18] The ghosts obtained were suspended in buffered saline (125 mM NaCl, 10 mM Na-phosphate buffer, and 1 mM EDTA, pH 7.4) at a concentration of 4 mg protein/ml. The protein content of the ghost suspensions was determined by the method of Lowry *et al.*[19] using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) at the standard.

### *Assay System for Hemolysis*

A 5% suspension of RBC in buffered saline was mixed with the same volume of azo compound solution, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) in buffered saline, so that the final mixture consisted of 2.5% RBC and 8.7 mM AAPH. The reaction mixture was shaken gently while being incubated at 37°C for 120 min,

and the extent of hemolysis was determined spectrophotometrically by the method reported previously.[15]

*Assay System for Oxidation of RBC Ghost Membranes*

The oxidation of RBC ghosts was induced by AAPH as follows.[20] An aliquot of the RBC ghost suspension was incubated in air at 37°C with the same volume of AAPH solution (the final mixture consisted of 2 mg protein/ml ghosts and 8.7 mM AAPH). The reaction conditions, such as the temperature and the concentration of the initiator, were determined so as to obtain appropriate rates of oxidation and disappearance of membrane reactants.

The rate of oxygen consumption in the reaction mixture was measured continuously using an automatic recording apparatus equipped with an oxygen electrode, Oxygraph Model 5/6H (Gilson Medical Electronics Inc., Middleton, WI). This provided an accurate measure of the rate of oxidation of the RBC ghosts.[21–23]

*Determination of Ghost Membrane Tocopherol*

Membrane tocopherol was extracted from the reaction mixtures with ethanol/hexane (2/5, v/v),[24] and levels were determined using high-performance liquid chromatography with electrochemical detection (Amperometric E-520, Irika Co., Kyoto).[25]

*Analysis of Ghost Membrane Lipids*

Membrane lipids were extracted from the reaction mixtures with isopropanol/chloroform (11/7, v/v),[26] and the fatty acid composition of the lipid extract was analyzed by gas-liquid chromatography with flame ionization detection (GC-8A, Shimadzu Co., Kyoto) after hydrolysis and esterification with HCl (3%)-methanol.[15]

*Statistical Analysis*

All results are expressed as the mean ± SD. Statistical significance was assessed using the unpaired Student's t-test. Linear regression analysis was performed using the least squares method.

## RESULTS

*Hemolysis of Cord and Adult RBC Induced by AAPH*

Figure 1 shows the development of lysis of cord and adult RBC as a result of membrane damage induced by AAPH. The onset of lysis of cord RBC was simultaneous with that of adult RBC, but the rate of lysis of cord RBC was faster than that of adult RBC. The α-tocopherol contents of cord and adult RBC were 179 ± 33 and 183 ± 10 μg/dl packed cells, respectively. Although the tocopherol content of cord RBC was almost the same as that of adult RBC, it appears that cord RBC are more susceptible to oxidative stress.

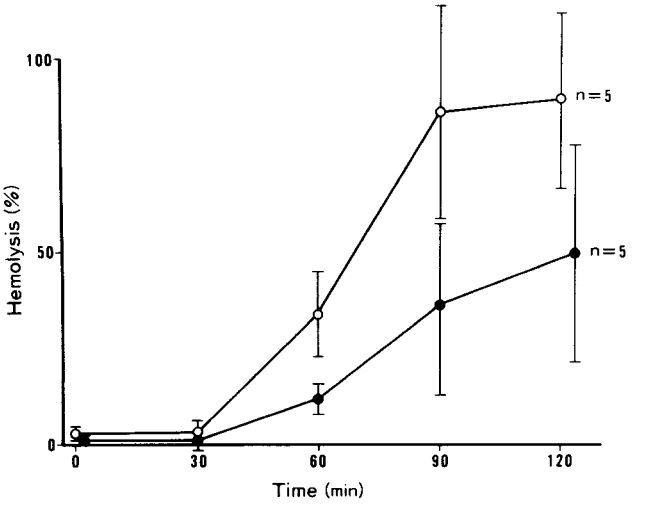


FIGURE 1 Lysis of cord and adult RBC induced by AAPH as a function of time. A suspension of RBC in buffered saline was mixed with AAPH (2.5% RBC, 8.7 mM AAPH) and incubated at 37°C with gentle shaking. Open circles indicate cord RBC and closed circles indicate adult RBC. Results are means ± SD of five experiments.

### *Oxygen Uptake by RBC Ghosts Reacted with AAPH*

Figure 2 shows the representative data of the oxidation of RBC ghosts initiated with AAPH at 37°C. When AAPH was added and the initiating radicals were generated at a constant rate in the aqueous phase, both oxygen and membrane α-tocopherol were consumed. Initially, the oxygen consumption was suppressed to form the induction period until tocopherol was exhausted. When the consumption of tocopherol ended, the induction period was over and a rapid oxidation occurred subsequently. The rate of oxygen uptake during and after the induction period was designated as $R_{inh}$ and $R_p$, respectively, and both were expressed in moles of oxygen consumed per litre per second (mol/L/s). The length of the induction phase was referred to as $t_{inh}$, and was expressed in seconds. Our findings were coincident with those of Yamamoto *et al.*[23]

### *Tocopherol Content and the Rate of Oxidation of Cord, Maternal, and Adult RBC Ghosts*

The initial tocopherol content (α- and γ-tocopherol), the length of the induction phase ($t_{inh}$) and the rate of oxygen uptake ($R_{inh}$, $R_p$) in the oxidation of cord, maternal, and adult RBC ghosts were shown in Table I. The tocopherol content and $t_{inh}$ were similar among the three types of ghosts. The $R_p$ value in cord ghosts was greater than that in adult ghosts, while the $R_{inh}$ values were not different among the three types of ghosts.

The kinetic chain length (KCL) is defined as the ratio of the propagation rate to the initiation rate and thus a longer KCL indicates longer chain propagation. AAPH constantly produce initiating radicals in an aqueous region and the number of radicals actually attacking membrane constituents may be constant.[20] One molecule

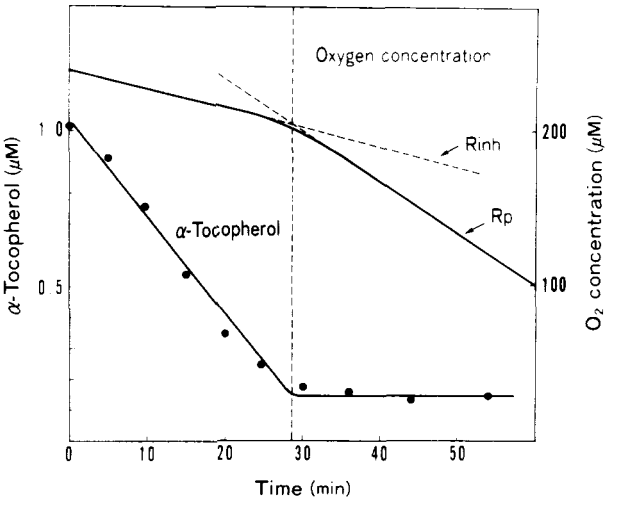


FIGURE 2 Oxygen uptake and α-tocopherol consumption during the oxidation of RBC ghosts initiated by AAPH. A ghost suspension in buffered saline was mixed with AAPH (2 mg protein/ml ghosts, 8.7 mM AAPH) and incubated at 37°C with stirring. During the reaction, the oxygen concentration in the reaction medium was continuously monitored and membrane α-tocopherol content was measured at the specific intervals. The rate of oxygen uptake during and after the induction period was designated as $R_{inh}$ and $R_p$, respectively.

of α-tocopherol is known to trap two molecules of radicals,[4,5] so the linear decrease in α-tocopherol in the ghosts during AAPH reaction indicating that initiating radicals are constantly scavenged with the depletion of membrane tocopherol. Therefore, the rate of initiation can be estimated on the basis of the tocopherol consumption rate. Accordingly, the ratio of oxygen uptake rate ($R_p$) to twice the tocopherol consumption rate gave the KCL value. As shown in Table I, the KCL was longer in cord ghosts than in adult ghosts, indicating that cord ghosts are more susceptible to oxidative stress.

*Relationship Between Total Tocopherol Content and the Length of the Induction Phase*

Vitamin E is the main chain-breaking lipid-soluble antioxidant in RBC membranes,[27] therefore, we subsequently plotted $t_{inh}$ against initial content of membrane tocopherol

TABLE I
Tocopherol content and the peroxidisability of three types of ghosts

| | Cord ($n$ = 10) | Maternal ($n$ = 7) | Adult ($n$ = 11) |
|---|---|---|---|
| Tocopherol (μmol/L) | | | |
| Total | 1.67 ± 0.47 | 1.37 ± 0.26 | 1.67 ± 0.18 |
| alpha | 1.54 ± 0.47 | 1.25 ± 0.23 | 1.49 ± 0.17 |
| gamma | 0.13 ± 0.13 | 0.12 ± 0.04 | 0.18 ± 0.04 |
| $t_{inh} \times 10^3$ (sec) | 2.02 ± 0.54 | 1.93 ± 0.39 | 2.09 ± 0.49 |
| $R_{inh}$ ($10^{-9}$ mol/L/sec) | 27.7 ± 7.1 | 24.6 ± 6.6 | 24.7 ± 4.4 |
| $R_p$ ($10^{-9}$ mol/L/sec) | 56.6 ± 7.1* | 49.9 ± 8.9 | 46.9 ± 7.1 |
| KCL | 27.5 ± 3.5* | 24.2 ± 4.3 | 22.7 ± 3.7 |

M ± SD, *$p < 0.05$ (vs. adult)

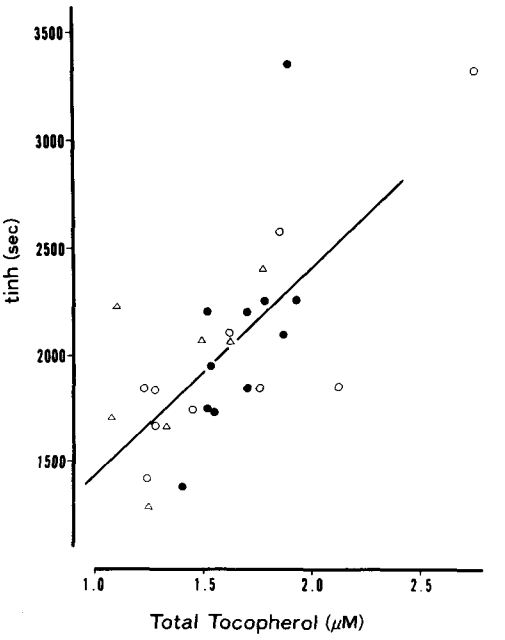


FIGURE 3 Relationship between the length of the induction phase and total tocopherol content in the membranes. In the oxidation of ghosts induced by AAPH, the oxygen consumption was suppressed to form the induction period until tocopherol was exhausted. The length of the induction phase was referred to as $t_{inh}$ and plotted against the initial tocopherol content for all three types of ghosts; open circles, triangles, and closed circles represent the data for cord, maternal, and adult RBC ghosts, respectively. Linear regression analysis was performed and a good correlation was observed ($r = 0.698$, $p < 0.05$, $n = 28$).

for all three types of ghosts. A good correlation was observed between them as shown in Figure 3.

### *Membrane Fatty Acid Composition and Active Bisallylic Hydrogen Content in the RBC Ghosts*

The rapid oxygen uptake ($R_p$) and long KCL immediately after the total depletion of membrane tocopherol suggested the occurrence of extensive chain propagation in the membrane lipids. It is generally accepted that polyunsaturated fatty acids (PUFA) may be the major substrates of oxidation. Therefore, the fatty acid composition of the RBC ghost membranes was examined. In Table II, the fatty acid content and the composition of the membrane lipids are shown per 2 mg of protein for the three types of ghosts. The fatty acid content of cord ghosts was greater than that of adult ghosts, while the corresponding amounts in maternal ghosts are variable. The cord ghosts were rich in PUFA, especially arachidonic acid (C20 : 4) and eicosatrienoic acid (C20 : 3), as compared with the other ghosts. From the PUFA composition and content of the three types of ghosts, the quantities of active hydrogen (active H) were calculated [for example, linoleic acid (C18 : 2) and arachidonic acid (C20 : 4) have 2 and 6 bisallylic hydrogen atoms, respectively]. The active H content may constitute another index of the peroxidizability of ghost membranes.[23] The active H content of

TABLE II
Membrane fatty acid constituents of cord, maternal, and adult RBC ghosts

| | Cord ($n$ = 10) | Maternal ($n$ = 7) | Adult ($n$ = 11) |
|---|---|---|---|
| Fatty acids ($\mu$mol/2 mg protein) | 3.74 ± 0.68* | 3.17 ± 1.49 | 2.24 ± 0.41 |
| Fatty acid composition (%) | | | |
| 14:0 | 0.7 ± 0.2 | 0.7 ± 0.2 | 0.6 ± 0.2 |
| 16:0 | 37.5 ± 2.9 | 34.9 ± 3.7 | 35.5 ± 9.3 |
| 16:1 | 0.7 ± 0.3 | 0.5 ± 0.2 | 0.2 ± 0.2 |
| 18:0 | 15.9 ± 2.4 | 13.2 ± 2.0 | 16.9 ± 0.6 |
| 18:1 | 10.2 ± 1.2 | 13.2 ± 1.3 | 13.8 ± 0.8 |
| 18:2 | 4.7 ± 0.8* | 12.7 ± 5.0 | 10.6 ± 0.9 |
| 20:3 | 2.4 ± 0.3* | 0.8 ± 0.4 | 0.8 ± 0.3 |
| 20:4 | 16.4 ± 2.1* | 11.1 ± 1.5 | 11.9 ± 1.7 |
| 22:0 | 0.5 ± 0.2 | 0.7 ± 0.4 | 0.5 ± 0.1 |
| 22:4 | 1.5 ± 0.5 | 1.1 ± 0.3 | 1.5 ± 0.2 |
| 22:6 unknown | 6.9 ± 1.3 | 7.7 ± 1.3 | 5.9 ± 1.5 |
| | 1.4 ± 0.5 | 2.5 ± 0.9 | 3.3 ± 1.1 |
| Active H | | | |
| (No.)[1] | 1.94 ± 0.23* | 1.79 ± 0.26 | 1.63 ± 0.25 |
| ($\mu$mol)[2] | 7.25 ± 1.53* | 5.60 ± 2.75 | 3.64 ± 0.79 |

M ± SD, *$p$ < 0.05 (vs. adult)
[1]Average number of active hydrogen atoms in one fatty acid molecule calculated from the fatty acid composition.
[2]Total number of active hydrogen atoms in 2 mg of RBC ghost protein.

cord ghosts was also larger than that of adult ghosts on the basis of both membrane lipids and proteins [see average number and total number of active hydrogen atoms shown in Table II].

### *Relationship Between the Active H Content and the Rate of Oxygen Uptake*

The relationships between the active H content and $R_{inh}$, $R_p$ are shown in Figure 4. The active H content of the ghosts correlated closely with $R_p$, but poorly with $R_{inh}$. This indicates that the suppression of chain propagation by membrane tocopherol was not greatly affected by the active H content and that the propagation rate was proportional to the amount of active H. The higher peroxidizability of cord ghosts, which were represented by their greater $R_p$ and KCL, is probably attributable to their larger active H content.

## DISCUSSION

It is well known that azo compounds, such as AAPH, generate free radicals constantly by thermal decomposition as shown below:

$$A\text{–}N{=}N\text{–}A \rightarrow A\cdot + N_2 + A\cdot$$

$$A\cdot + O_2 \rightarrow AO_2\cdot$$

$$AO_2\cdot \rightarrow \text{attacks membranes}$$

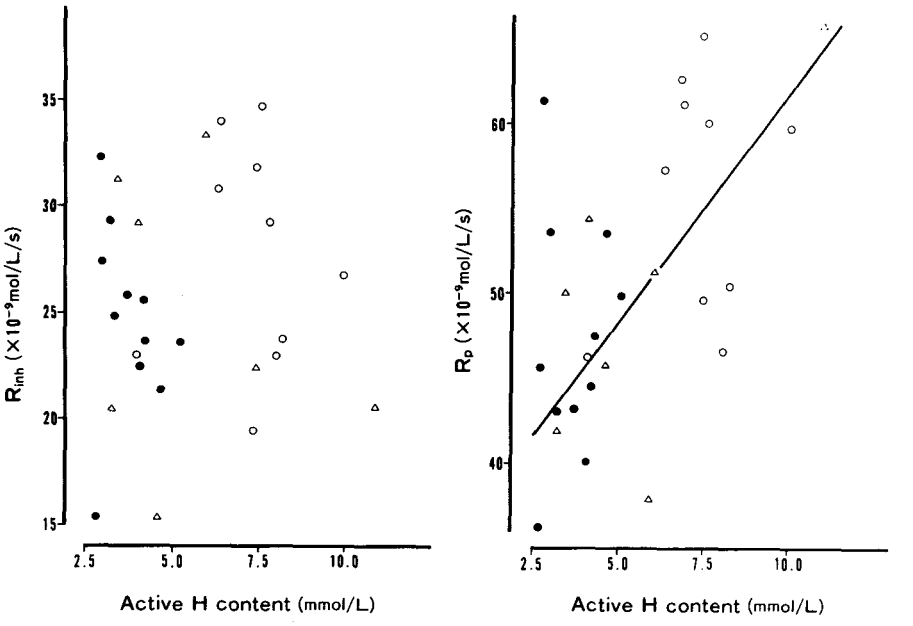


FIGURE 4 Relationship between the active H content and the rate of oxygen uptake. The amount of bisallylic hydrogen atoms (active H content) in the ghost suspension was calculated from its PUFA composition and content. Open circles, triangles, and closed circles represent the data for cord, maternal, and adult RBC ghosts, respectively. The active H content correlated poorly with $R_{inh}$ (left) and closely with $R_p$ (right; $r = 0.553$, $p < 0.05$, $n = 28$).

where $A$ is $HCl \cdot HN{=}C(NH_2)C(CH_3)_2$ for AAPH. Free radicals produced from AAPH rapidly react with oxygen to give peroxyl radicals, which attack the membrane constituents and abstract bisallylic hydrogen atoms to produce lipid radicals. The lipid peroxyl radicals then induce a sequence of propagation reactions that give rise to lipid hydroperoxides.[2,23] This chain oxidation of RBC membrane constituents initiated by AAPH eventually leads to hemolysis, and hemolysis was inhibited by membrane α-tocopherol.[15,28] Our current kinetic study showed a greater oxygen uptake rate in the propagation ($R_p$) and a longer KCL in cord ghosts as compared with those in adult ghosts. These differences were attributable to the constituents of the membrane lipids, especially PUFA, and not to the tocopherol content. The greater active H content of cord ghosts seemed to result in an increased susceptibility of their membranes to oxidative stress despite an adequate tocopherol content.

A higher level peroxidation may occur also in other neonatal biomembranes under conditions of oxidative stress in comparison with adult membranes, as was shown for RBC ghosts in this study. There were a greater oxygen uptake rate ($R_p$) and longer KCL in cord ghosts compared with adult ghosts, even though there were no differences in $R_{inh}$, $t_{inh}$, and tocopherol content. Therefore, neonatal cell membranes in general may be highly susceptible to oxidative stress. For neonatal membranes to have a higher peroxidizability than that of adult membranes under the same oxidative conditions, a higher membrane tocopherol content would be required. Figure 5 shows that as the tocopherol content increases from A to B the $t_{inh}$ in neonatal membranes is prolonged from $t_1$ to $t_2$, so that at the arbitrary end point of propagation (E) the

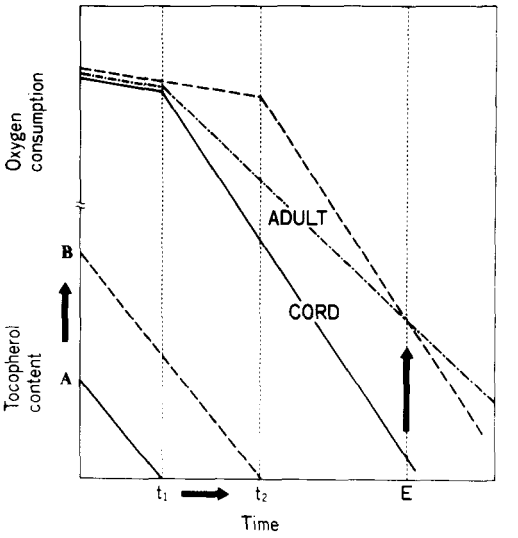


FIGURE 5 Changes in the peroxidizability of RBC ghosts with increasing membrane tocopherol. The lines show the representative consumption curves of oxygen and tocopherol during AAPH-induced oxidation of RBC ghosts.

peroxidizability of neonatal membranes would be comparable to that of adult membranes. Therefore, even though the tocopherol content of neonatal and adult membranes is similar, more tocopherol may be required for the protection of neonatal membranes under conditions of oxidative stress, such as hyperoxia induced by mechanical ventilation or inflammation brought by infection.

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