Biochimica et Biophysica Acta 861 (1986) 311–318
Elsevier

BBA 73300

A change in the lipid fluidity of the porcine intestinal brush-border membranes by lipid peroxidation. Studies using pyrene and fluorescent stearic acid derivatives

Takao Ohyashiki, Teruyuki Ohtsuka and Tetsuro Mohri

Department of Physiological Chemistry, School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa, Ishikawa 920-11 (Japan)

(Received 4 April 1986)

Key words: Lipid peroxidation; Membrane fluidity; Pyrene fluorescence; Fluorescent fatty acid; (Porcine intestinal brush-border membrane)

The effect of lipid peroxidation on the lipid fluidity of porcine intestinal brush-border membranes was examined by measuring the rotational mobility and the accessibility to fluorescence quenchers (CH_3COOTl, $CuSO_4$ and KI) of pyrene or *n*-(9-anthroyloxy)stearic acid ($n = 2$ or 12) in the membranes. The harmonic mean of the rotational relaxation times of pyrene increased and the rate constants, k_q, of the quenching reaction of pyrene and 2-(9-anthroyloxy)stearic acid incorporated in the membrane lipids decreased upon lipid peroxidation, indicating reduction of the lipid fluidity of the membranes by lipid peroxidation. In addition, the k_q value of the reaction of 2-(9-anthroyloxy)stearic acid in the membranes with Cu^{2+} decreased in proportion to the amount of the products of lipid peroxidation. On the other hand, the k_q value of the reaction of 12-(9-anthroyloxy)stearic acid with Cu^{2+} or I^- was unaffected by lipid peroxidation. Based on these results, a localized change in the lipid fluidity of the membranes in association with lipid peroxidation has been discussed.

Introduction

Lipid peroxidation of the biological membranes has been attributed to exert a primary effect in the process of a variety of pathological events including the aging of cells [1]. The biological membranes are readily susceptible to peroxidative attack because they are rich in highly unsaturated fatty acids as the constituents of lipids.

It has been believed that lipid-lipid and lipid-protein interactions in the biological membranes play a major role in their functions [2,3]. Therefore, it must be vary important to study the effects of lipid peroxidation on these dynamic features in the membrane components in order to understand the molecular mechanism(s) mediating the modification of the membrane functions induced by the lipid peroxidation.

Recently, several investigators have demonstrated by different techniques including fluorescence polarization [4–6], electron spin resonance [7] and X-ray diffraction [8] that lipid peroxidation of the liposome membranes results in the decrease of their lipid fluidity. On the other hand, several biochemical changes induced by lipid peroxidation have also been reported in biological membranes; reduction of membrane lipid fluidity [9–12] and inactivation of membrane-bound enzymes [13,14].

In the previous paper [15] we reported that the

Correspondence address: Department of Physiological Chemistry, School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa, Ishikawa 920-11, Japan.

brush-border membranes from porcine small intestine is peroxidized by incubating them with ferrous ions in the presence of dithiothreitol and that the formation of thiobarbituric acid-reactive substances is dependent on the nature of the membrane surface charges. In the present study we attempted to explore by using several fluorescent dyes with different solubility and distribution, pyrene and 2- or 12-(9-anthroyloxy)stearic acid, the topographic changes of the lipid fluidity in the porcine intestinal brush-border membranes upon their lipid peroxidation.

Materials and Methods

Preparation of membrane vesicles. Brush-border membrane vesicles were prepared from porcine small intestine by the calcium-precipitation method as described in our previous paper [16] and suspended in 10 mM Tris-maleate buffer (pH 6.85). Protein concentration was assayed by procedure of Lowry et al. [17] using bovine serum albumin as standard.

Chemicals. Pyrene and 2- or 12-(9-anthroyloxy)stearic acid were purchased from Wako Pure Chemical Co. and Molecular Probes Co., respectively. The stock solutions of these fluorescent dyes (1 mM of each) were made by dissolving in ethanol (pyrene) and methanol (the others). All other reagents used were of the highest purity obtainable from commercial sources.

Labeling of membrane vesicles with fluorescent dyes. A suspension of the membrane vesicles (1.5–2.0 mg protein/ml) was incubated with 2 μM pyrene or 10 μM *n*-(9-anthroyloxy)stearic acid ($n = 2$ or 12) in 10 mM Tris-maleate buffer (pH 6.85) at 0°C for 30 min. The final concentration of ethanol or methanol used as a solvent for each dye in the reaction mixture was 0.33%. The reaction was terminated by dilution of the reaction mixture with a large volume of the same buffer and centrifuged at $25\,000 \times g$ for 20 min. The pellets were washed twice with the same buffer and resuspended in 10 mM Tris-maleate buffer (pH 6.85).

Peroxidation of the membrane lipids. Lipid peroxidation was performed by the incubation of a suspension of the membrane vesicles (2.5 mg protein/ml) with 100 μM ascorbic acid and 10 μM $FeSO_4$ in 10 mM Tris-maleate buffer (pH 6.85) at 37°C for 30 min, unless otherwise specified. The reaction was terminated by addition of 3-*tert*-butyl-4-hydroxyanisole at 5 mM to the reaction mixture. It is recognized that the membrane lipid peroxidation under the conditions mentioned above is completely arrested by this concentration of 3-*t*-butyl-4-hydroxyanisole [15]. The reaction mixture was then diluted with a large volume of ice-cold 10 mM Tris-maleate buffer (pH 6.85) and centrifuged at $25\,000 \times g$ for 20 min. The pellets obtained were washed twice with and suspended in the same buffer. The amount of thiobarbituric acid-reactive substances formed during the reaction was determined fluorometrically using 1,1,3,3-tetraethoxypropane as a standard and expressed as equivalents of malondialdehyde as described previously [15].

Fluorescence measurements. Steady-state fluorescence measurements were performed on a Hitachi fluorescence spectrophotometer MPF-4 equipped with a rhodamine B quantum counter. The sample compartment was maintained at 25°C by circulating water through the cell holder unless otherwise specified. The excitation and emission wavelengths used for fluorescence measurements of pyrene and *n*-(9-anthroyloxy)stearic acids were 340 and 392 nm, and 360 and 435 nm, respectively.

The degree of fluorescence polarization, P, was calculated according to the equation: $P = (I_V - I_H)/(I_V + I_H)$, where I_V and I_H are the fluorescence intensities detected by a polarizer oriented parallel and perpendicular, respectively, to the direction of polarization of the excitation beam. The harmonic mean of the rotational relaxation times, ρ_h, of pyrene molecules in the membranes was determined from the slope of a $1/P$ vs. T/η plot and the fluorescence lifetime, τ, using the following equation [18]:

$$\rho_h = \left(\frac{1}{P_0} - \frac{1}{3}\right)\frac{3\tau}{\text{the slope}} \cdot \frac{\eta}{T}$$

where P_0, η and T denote the limiting polarization in the absence of rotational motion, the solvent viscosity and the absolute temperature, respectively.

Fluorescence lifetime measurements were car-

ried out an Ortec PRA-3000 photon counting nanosecond fluorometer (Photochemical Research Associates Inc., Ontario, Canada). Data were analysed using a Digital MINC-11 computer system (Digital Equipment Co., MA, U.S.A.).

Quenching experiments were performed by a titration procedure with small amounts of concentrated quencher solutions at 25°C. The stock solutions of the quenchers (CH_3COOTl, $CuSO_4$ and KI) were used within a few days after preparation. A collisional quenching of fluorescence can be described by the Stern-Volmer equation [19].

$$I_0/I = 1 + K_Q[Q]$$

where

$$K_Q = k_q\tau_0$$

In these equations, I_0 and I are the fluorescence intensities in the absence and presence of quencher, respectively, K_Q is the quenching constant, τ_0 is the fluorescence lifetime in the absence of quencher, k_q is the rate constant for the quenching reaction and [Q] is the concentration of quencher.

When the quenching does not follow the simple Stern-Volmer law, the quenching parameters were determined with the following modified equation proposed by Lehrer [20]:

$$I_0/\Delta I = (1/f_a K_Q[Q]) + (1/f_a)$$

where ΔI is the change in the fluorescence intensity due to addition of a given concentration of quencher and f_a is the fractional maximum accessible probe fluorescence, which is associated with the accessibility of the probe to quencher.

Results and Discussion

Time course of lipid peroxidation by ascorbic acid plus Fe^{2+}

Fig. 1 shows the time course of lipid peroxidation in the porcine intestinal brush-border membranes with 100 μM ascorbic acid plus 10 μM Fe^{2+} at 37°C in 10 mM Tris-maleate buffer (pH 6.85)

A time-dependent increase of malondialdehyde in the presence of ascorbic acid and Fe^{2+}, while no significant increase of it was observed during 120 min of incubation when both of these agents were excluded.

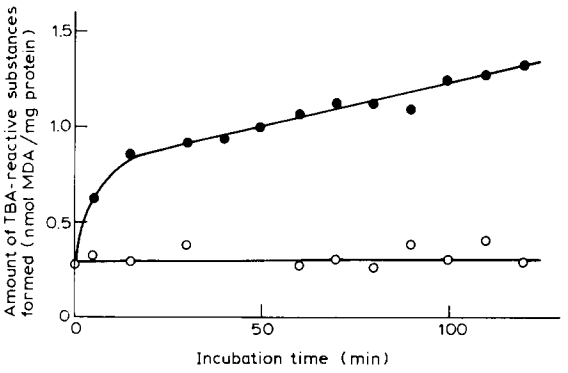

Fig. 1. The time course of lipid peroxidation of the membranes upon addition of ascorbic acid and Fe^{2+} in 10 mM Tris-maleate buffer (pH 6.85) at 37°C. Membrane protein concentration was 0.51 mg/ml. Symbols, ● and ○, represent the systems in the presence and absence (control) of ascorbic acid (100 μM) and Fe^{2+} (10 μM), respectively. TBA, thiobarbituric acid; MDA, malondialdehyde.

Temperature dependence of fluorescence polarization

We explored the temperature dependence of fluorescence polarization of the fluorophores incorporated in the membranes to monitor the influence of lipid peroxidation on the physical state of membrane components, assuming that the degree of polarization of the fluorophore in solution depends on the thermal rotations of the transition moment of fluorescence emission [21,22].

Fig. 2 shows the temperature-dependence profiles of the polarization of the fluorescence of pyrene in the control and peroxidized membranes. The degrees of the polarization in these systems decreased with increasing temperature in a monophasic manner over a temperature range from 15 to 47°C, and the slope in the Perrin plot was lower in the peroxidized membranes, suggesting restriction of the temperature-dependent movement of pyrene molecules as a result of lipid peroxidation [21,22].

In order to estimate quantitatively the magnitude of movement of pyrene molecules in the membranes, the harmonic mean of the rotational

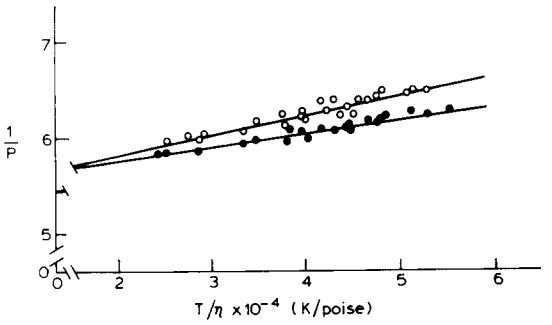

Fig. 2. Temperature dependence of the fluorescence polarization of pyrene-labeled membranes subjected to lipid peroxidation or not. Membrane protein concentration was 0.10 mg/ml. Temperature was varied from 15 to 47°C. ○, control membranes; ●, peroxidized membranes. Other experimental conditions were the same as described in the legend to Fig. 1.

relaxation times, ρ_h, of the dye in the membranes was calculated from the slope of the Perrin plot and the independently measured fluorescence lifetime, τ. The ρ_h and τ values for the control and peroxidized membranes are summarized in Table I.

The fluorescence decay curve of membrane-bound pyrene molecules in the control did not fit single exponential function (Fig. 3). This suggests that pyrene molecules locate in two or more different environments in the bilayer of the membranes [23]. We assumed, therefore, that there are two components in the decaying process of the fluorescence to analyze its lifetime. One of them was shown, by a computer fitting, to have a lifetime 177 ± 2.7 ns, accounting for 88% of the total fluorescence, and the other lifetime 6.4 ± 0.04 ns, accounting for the remaining 12% (Table I). By lipid peroxidation of the membranes these τ values were not changed, but the ρ_h value increased greatly as shown in Table I. From these results it is inferred that the decrease of the slope in the Perrin plot of membrane-bound pyrene by lipid peroxidation is not attributed to the change in the excited-state lifetime but solely to an increase of structural constraint around the probe molecules in the lipid bilayers of the membranes. On the other hand, no thermal dependence of polarization was found with *n*-(9-anthroyloxy)stearic acids ($n = 2$ and 12) in the control and peroxidized membranes (data not shown). This lack of response may be due to their short fluorescence lifetimes in the membranes compared with the rate of rotational movement of the dye molecules (Table II).

TABLE I

FLUORESCENCE LIFETIME, LIMITING POLARIZATION (P_0) AND HARMONIC MEAN OF ROTATIONAL RELAXATION TIMES (ρ_h) OF PYRENE MOLECULES IN THE CONTROL AND PEROXIDIZED MEMBRANES

The ρ_h values were calculated from the data shown in Fig. 2 using the τ_2 value in each system.

System	P_0	Lifetime (ns) *		ρ_h (μs)
		τ_1	τ_2	
Control	0.182	6.4 ± 0.44	177 ± 2.7	4.35
Peroxidized	0.182	7.8 ± 0.95	177 ± 3.0	6.61

* Values are expressed as mean ± S.D. for three membrane preparations.

Fluorescence quenching studies

Since the dynamic quenching of fluorescence by soluble quenchers is dependent on diffusion-controlled process [19], the fluorescence quenching measurements provide the information about the solvent accessibility to fluorophores embedded in the membranes.

As can be seen in Fig. 4, quenching of the fluorescence of pyrene in the membranes by Tl^+ and I^- does not follow the simple Stern-Volmer law; the intercept on the ordinate is above zero. This suggests that the probe molecules are arranged within the lipid bilayers of the membranes so that only a proportion of the molecules is accessible to the quenchers.

The modified Stern-Volmer plots of the fluorescence of pyrene-labeled control and peroxidized membranes for these quenchers are shown in Fig. 5. The plots of $I_0/\Delta I$ vs the reciprocal of the quencher concentration showed straight lines over the concentration ranges of the quenchers tested. The quenching parameters are summarized in Table III.

The rate constant, k_q, of the quenching of pyrene fluorescence by Tl^+ and I^- definitely decreased upon lipid peroxidation of the membranes, indicating reduction of the accessibility of these quenchers to pyrene molecules incorporated in the membrane lipids. The f_a values for these

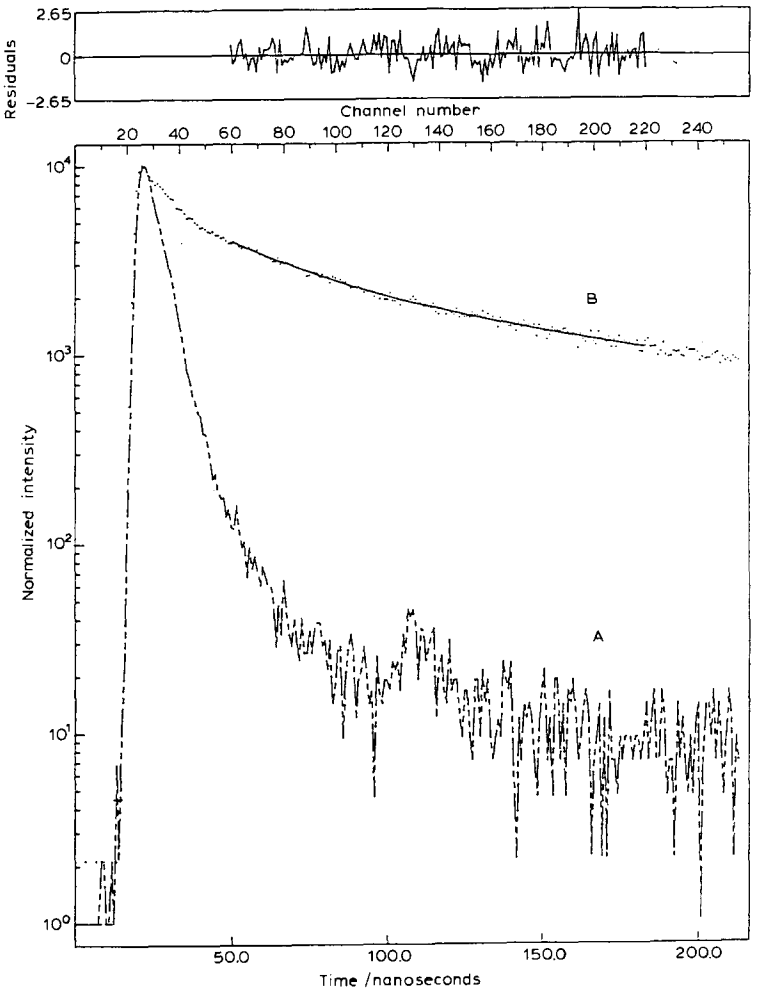

Fig. 3. A specimen of the fluorescence decay curve of pyrene-labeled membranes in 10 mM Tris-maleate buffer (pH 6.85) at 25°C. Membrane protein concentration was 0.100 mg/ml. Excitation wavelength used was 340 nm. Curves A and B represent excitation pulse and fluorescence of pyrene-labeled membranes, respectively. The solid line in the curve B shows the fitting curve by the non-linear least-square method. The figure of the residuals is shown above top of the graph.

TABLE II

FLUORESCENCE LIFETIMES OF *n*-(9-ANTHROYLOXY)STEARIC ACID DERIVATIVES ($n = 2$ AND 12) IN THE CONTROL AND PEROXIDIZED MEMBRANES

Membrane protein concentration was 0.200 mg/ml. Other experimental conditions were the same as described in the legend to Fig. 1.

System	Lifetime (ns) *			
	2AS **		12AS **	
	τ_1	τ_2	τ_1	τ_2
Control	2.46 ± 0.34	9.40 ± 0.18	2.66 ± 0.16	11.1 ± 0.33
Peroxidized	2.66 ± 0.32	10.00 ± 0.24	2.53 ± 0.17	11.5 ± 0.33

* Values are expressed as mean ± S.D. for three membrane preparations.

** 2AS and 12AS; 2- and 12-(9-anthroyloxy)stearic acids, respectively.

quenchers in the control membranes, 0.716 for Tl^+ and 0.337 for I^-, allow us to estimate that about 72 and 34% of pyrene molecules in the membranes are susceptible to Tl^+ and I^- ions, respectively. These f_a values were scarcely changed by lipid peroxidation. Therefore it seems that a decrease of the k_q value for Tl^+ and I^- by lipid peroxidation is mainly attributed to an enhancement of collisional efficiency between the quencher molecules and fluorophores embedded in the membrane lipids.

We have next examined the variation in the responsiveness of fluorophore to a quencher at different depths of the lipid bilayers of the control and peroxidized membranes. For this purpose a set of *n*-(9-anthroyloxy)stearic acids ($n = 2$ and

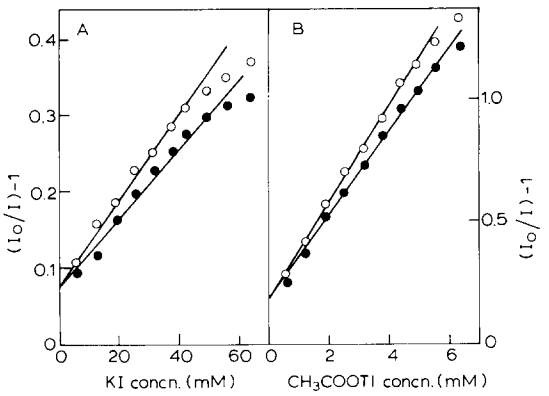

Fig. 4. Stern-Volmer plots of fluorescence intensities of pyrene-labeled membranes. Membrane protein concentration was 0.08 mg/ml. The concentrations of KI and CH_3COOTl were varied from 6.62 to 62.5 and from 0.662 to 6.25 mM, respectively. Other experimental conditions and symbols were the same as described in the legend to Fig. 2. (A) KI; (B) CH_3COOTl. 25°C.

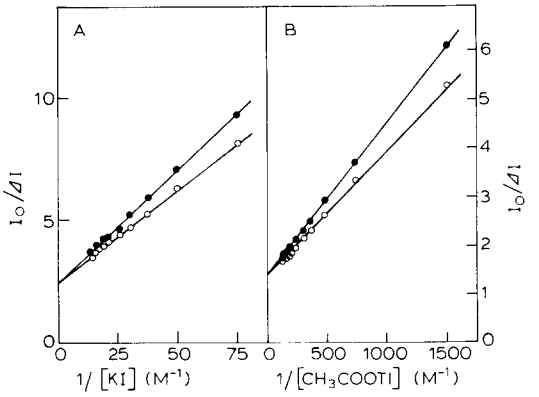

Fig. 5. Modified Stern-Volmer plots of fluorescence intensities of pyrene-labeled control (○) and peroxidized (●) membranes. The data were obtained from Fig. 4. (A) KI; (B) CH_3COOTl.

12), in which anthroic acid is linked to position 2 or 12 of stearic acid by an ester bond, were incorporated in the membranes. These probes are expected to be a sensor of the local fluidity within the lipid bilayers, because the fluorescent core of these dyes would monitor the molecular flexibility at different depths in the lipid bilayers [24–26] if the dyes could be oriented in the membranes at an angle against the plane of them. We have used $CuSO_4$ as a quencher in addition to KI in this experiment, because the Cu^{2+} ion is an effective quencher of the fluorescence of 9-anthroyloxy stearate [24].

Table IV shows the quenching parameters of the fluorescence of 2- and 12-(9-anthroyloxy)stearic acids attached to the membranes for $CuSO_4$ and KI. The quenching of the fluorescence of *n*-(9-anthroyloxy)stearic acids in the membranes by Cu^{2+} of I^- again did not follow the simple Stern-Volmer law. Therefore, the quenching parameters for Cu^{2+} and I^- in the control and peroxidized membranes were determined by calculation the modified Stern-Volmer plots.

The k_q values of the quenching of 2-(9-anthroyloxy)stearic acid by Cu^{2+} and I^- were all decreased by lipid peroxidation similarly to those of pyrene fluorescence. In addition, there is a good correlation between the degrees of the decrease of the k_q value of 2-(9-anthroyloxy)stearic acid for Cu^{2+} and the amounts of peroxidative products expressed as malondialdehyde with various concentrations of ascorbic acid (Fig. 6). In contrast, the quenching of 12-(9-anthroyloxy)stearic acid

TABLE III

QUENCHING PARAMETERS OF PYRENE-LABELED MEMBRANES WITH Tl^+ and I^-

Membrane protein concentration was 0.08 mg/ml. The concentrations of CH_3COOTl and KI were varied from 0.662 to 6.25 and 6.62 to 62.5 mM, respectively. Other experimental conditions were the same as described in the legend to Fig. 1.

Quenchers	$K_Q\ (M^{-1})$ *		$k_q\ (M^{-1}\cdot s^{-1})$		f_a*	
	control	peroxidized	control	peroxidized	control	peroxidized
CH_3COOTl	526 ±6.57	439 ±8.49	$2.97\cdot10^9$	$2.48\cdot10^9$	0.716±0.03	0.698±0.04
KI	31.9±0.82	27.9±1.84	$0.18\cdot10^9$	$0.15\cdot10^9$	0.337±0.02	0.333±0.03

* Values are expressed as mean ± S.D. for three membrane preparations.

TABLE IV

QUENCHING PARAMETERS OF *n*-(9-ANTHROYLOXY)STEARIC ACIDS IN THE CONTROL AND PEROXIDIZED MEMBRANES FOR Cu^{2+} AND I^-

Membrane protein concentration was 0.08 mg/ml. The $CuSO_4$ concentration was varied from 6.62 to 62.5 μM. Other experimental conditions were the same as described in the legend to Table III. Values for $CuSO_4$ are given as mM^{-1}, and those for KI as M^{-1}.

Quenchers		K_Q*		k_q ($M^{-1} \cdot s^{-1}$)		f_a*	
		control	peroxidized	control	peroxidized	control	peroxidized
$CuSO_4$	2AS-membranes	76.0 ± 2.35	65.8 ± 2.46	$7.60 \cdot 10^{12}$	$6.78 \cdot 10^{12}$	0.773 ± 0.01	0.773 ± 0.01
	12AS-membranes	26.7 ± 1.23	26.3 ± 0.96	$2.41 \cdot 10^{12}$	$2.27 \cdot 10^{12}$	0.761 ± 0.04	0.761 ± 0.04
KI	2AS-membranes	12.4 ± 0.40	8.74 ± 1.09	$1.24 \cdot 10^{9}$	$0.90 \cdot 10^{9}$	0.307 ± 0.02	0.302 ± 0.03
	12AS-membranes	3.56 ± 0.04	3.55 ± 0.02	$0.32 \cdot 10^{9}$	$0.31 \cdot 10^{9}$	0.269 ± 0.02	0.267 ± 0.01

* Values are expressed as mean ± S.D. for three membrane preparations.
** 2AS and 12AS; 2- and 12-(9-anthroyloxy)stearic acid, respectively.

fluorescence in the membranes by Cu^{2+} or I^- was scarcely influenced by lipid peroxidation (Table IV). Similar results were also obtained in the quenching experiments using Tl^+. In this case, the

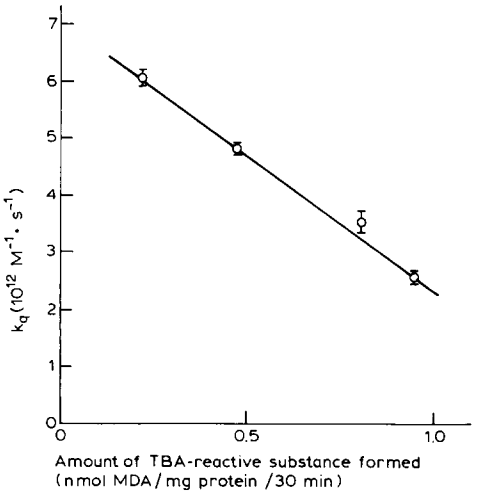

Fig. 6. Relationship between the quenching rate constant of 2-(9-anthroyloxy)stearic acid fluorescence in the membranes for Cu^{2+} and the amount of malondialdehyde (MDA) formed. The lipid peroxidation was performed by incubating the membrane vesicles with various concentrations of ascorbic acid (0, 5, 30 and 100 μM) in the presence of 10 μM Fe^{2+} at 37°C for 30 min. The reaction was terminated by addition of 5 mM 3-*t*-butyl-4-hydroxyanisole as a final concentration and the amount of thiobarbituric acid (TBA)-reactive substances formed was determined. Membrane protein concentrations used for measurement of malondialdehyde formation and k_q determination of Cu^{2+} were 0.51 and 0.08 mg/ml, respectively. Other experimental conditions were the same as described in the legend to Fig. 5. Values are expressed as mean ± S.D. for three membrane preparations.

k_q values of 2-(9-anthroyloxy)stearic acid in the control and peroxidized membranes were $34.4 \cdot 10^9$ and $25.2 \cdot 10^9$ $M^{-1} \cdot s^{-1}$, respectively. In the 12-(9-anthroyloxy)stearic acid-labeled membranes however, the k_q values for Tl^+ were almost the same in the both membrane systems ($0.33 \cdot 10^9$ and $0.31 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ for the control and peroxidized membranes, respectively).

From these results, it could be considered that the lipid peroxidation of the brush-border membranes results in a marked reduction of the lipid fluidity and its influence reaches up to the lipid organization near the membrane surface, assuming that the molecules of these stearate derivatives are embedded in the membrane lipid bilayers orientating their carboxy groups toward the surface. In addition, the discrepancy of responses in pyrene and 12-(9-anthroyloxy)stearic acid fluorescence in the membranes for I^- and Tl^+ seems due to difference of distribution and/or configuration of these probes in the membrane lipids, although the exact reason is not clear at present.

References

1 Tappel, A.L. (1973) Fed. Proc. 32, 1870–1874
2 Fox, C.F. (1975) in Biochemistry of Cell Walls and Membranes (Fox, C.F., ed.), Vol. 2, pp. 279–306, University Park Press, Baltimore, MD
3 Tada, M., Yamamoto, T. and Tonomura, Y. (1978) Physiol. Rev. 58, 1–79
4 Barrow, D.A. and Lentz, B.R. (1981) Biochim. Biophys. Acta 645, 17–23

5 Galanopoulou, D., Williams, W.P. and Quinn, P.J. (1982) Biochim. Biophys. Acta 713, 315–323
6 Sunamoto, J., Baba, Y., Iwamoto, K. and Kondo, H. (1985) Biochim. Biophys. Acta 833, 144–150
7 Nagatsuka, S. and Nakazawa, T. (1982) Biochim. Biophys. Acta 691, 171–177
8 Pauls, K.P. and Thompson, J.E. (1980) Nature 283, 504–506
9 Rice-Evans, C. and Hochstein, P. (1981) Biochem. Biophys. Res. Commun. 100, 1537–1542
10 Yamaguchi, T., Fujita, Y., Kuroki, S., Ohtsuka, K. and Kimoto, E. (1983) J. Biochem. 94, 376–386
11 Gut, J., Kawato, S., Cherry, R.J., Winterhalter, K.H. and Richter, C. (1985) Biochim. Biophys. Acta 817, 217–228
12 Rice-Evans, C., Baysal, E., Pashby, D.P. and Hochstein, P. (1985) Biochim. Biophys. Acta 815, 426–432
13 Plaa, G.L. and Witschi, H. (1976) Annu. Rev. Pharmacol. Toxicol. 16, 125–141
14 Chance, B., Sies, H. and Boveris, A. (1979) Physiol. Rev. 59, 527–605
15 Ohyashiki, T., Koshino, M., Ohta, A. and Mohri, T. (1985) Biochim. Biophys. Acta 812, 84–90
16 Ohyashiki, T., Takeuchi, M., Kodera, M. and Mohri, T. (1982) Biochim. Biophys. Acta 688, 16–22
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
18 Ohyashiki, T., Taka, M. and Mohri, T. (1985) J. Biol. Chem. 260, 6857–6861
19 Stern, O. and Volmer, M. (1919) Z. Phys. 20, 183–188
20 Lehrer, S.S. (1971) Biochemistry 10, 3254–3263
21 Weber, G. (1952) Biochem. J. 51, 145–155
22 Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394
23 Chen, L.A., Dale, R.E., Roth, S. and Brand, L. (1977) J. Biol. Chem. 252, 2163–2169
24 Thulborn, K.R. and Sawyer, W.H. (1978) Biochim. Biophys. Acta 511, 125–140
25 Thulborn, K.R., Tilley, L.M., Sawyer, W.H. and Treloar, F.E. (1979) Biochim. Biophys. Acta 558, 166–178
26 Tilley, L.M., Thulborn, K.R. and Sawyer, W.H. (1979) J. Biol. Chem. 254, 2592–2594