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The use of *cis*-parinaric acid to determine lipid peroxidation in human erythrocyte membranes. Comparison of normal and sickle erythrocyte membranes

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The recently developed parinaric acid assay is shown to offer possibilities for studying peroxidation processes in biological membrane systems. Taking the human erythrocyte membrane as a model, several initiating systems were investigated, as well as the effect of residual hemoglobin in ghost membrane preparations. The effectivity of a radical generating system appeared to be strongly dependent upon whether radicals are generated at the membrane level or in the water phase. Thus, cumene hydroperoxide at concentrations of 1.0–1.5 mM was found to be a very efficient initiator of peroxidation in combination with submicromolar levels of hemin-Fe^{3+} as membrane-bound cofactor. In combination with cumene hydroperoxide, membrane-bound hemoglobin appeared to be about 6-times more effective in promoting peroxidation than hemoglobin in the water phase. Results comparing the behaviour of normal and sickle erythrocyte ghost suspensions in the peroxidation assay suggest that the increased oxidative stress on sickle erythrocyte membranes could be due to enhanced membrane binding of sickle hemoglobin, but also partly to a characteristically higher capability of sickle hemoglobin to promote peroxidation. The order of peroxidation-promoting capabilities that could be derived from the experiments was hemin > sickle hemoglobin > normal hemoglobin.

Introduction

Oxygen free radicals are potentially dangerous species for living cells. In vivo they may originate from several intracellular and extracellular sources, including normal biological processes [1]. Some oxygen radicals are capable of entering a membrane and initiating a complex process of radical chain reactions by which polyunsaturated fatty acyl chains of membrane lipids are oxidatively degraded. This process, known as lipid peroxidation, is the subject of intensive research, as it is implicated in many pathological conditions [2,3,14].

Several methods have been developed for the determination of lipid peroxidation, each of which

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is dependent upon one specific feature of peroxidation and has its own characteristic advantages and disadvantages [4–6]. In a preceding paper [7] we have described a new method, which can monitor the peroxidation process in its initial stages. In this new method a fluorescent polyunsaturated fatty acid, parinaric acid (9,11,13,15-octadecatetraenoic acid), is used as a membrane probe for peroxidation. The basis for this fluorescent peroxidation assay is provided by the special structure of the parinaric acid molecule, its conjugated double bond system giving rise to the fluorescent properties as well as susceptibility to peroxidation. Peroxidation of the probe molecules is indicated by a decrease in fluorescence, which can be measured directly and sensitively. This method offers an opportunity to monitor the degradation of one particular polyunsaturated fatty acid in a sensitive, direct and continuous assay. The principle features of the parinaric acid method have been outlined for model membrane systems, and a correlation with existing methods for detecting peroxidation has been demonstrated [7].

The erythrocyte membrane is a biological membrane under constant oxidative stress, due to a number of factors related to the characteristic structure and function of the erythrocyte [8]. One of the factors involved is hemoglobin, which is present in large amounts within the erythrocyte. Hemoglobin, as well as its oxidative breakdown products, may stimulate lipid peroxidation [9,10]. Sickle cell anemia is a genetic disease, characterised by an abnormal hemoglobin which, under certain conditions, polymerises and causes the erythrocyte to become sickle shaped. In addition to an out-of-balance cytosolic antioxidant system [11,12] and abnormally low membrane vitamin E levels [13], it appears that the altered properties of the sickle hemoglobin are also an important factor with respect to the increased susceptibility to lipid peroxidation noted in sickle erythrocytes [14,15].

In the present study, we report the application of the parinaric acid method in peroxidation experiments with the human erythrocyte membrane. Several peroxidation initiating systems are investigated and their effects compared. Furthermore, parinaric acid peroxidation in ghost membranes is determined as a function of the amount of hemoglobin present. Following identical procedures of ghost preparation, results are compared for normal and sickle erythrocytes.

Materials and Methods

Materials

cis-Parinaric acid was purchased from Molecular Probes (Junction City, OR, U.S.A.). $CuSO_4 \cdot 5H_2O$ (p.a. grade) was obtained from U.C.B. (Brussels, Belgium) and $FeCl_3 \cdot 6H_2O$ (p.a. grade) from Merck (Darmstadt, F.R.G.). Cumene hydroperoxide and *tert*-butyl hydroperoxide were received as a kind gift from Akzo Chemie (Amersfoort, The Netherlands).

Hemin (bovine) was obtained from Sigma (St. Louis, MO, U.S.A.), and was dissolved in water containing NaOH. Diluted hemin solutions containing 100 μM hemin at a pH of 12.0 were used within one week.

Erythrocytes

Fresh human erythrocytes, both from healthy individuals and from sickle cell anemia patients, were obtained from volunteers by venipuncture after obtaining informed consent. Acid-citrate-dextrose was used as anticoagulant [16]. Cells were centrifuged for 10 min at $1300 \times g$ or $1000 \times g$ for normal and sickle erythrocytes, respectively, and washed three times with an isotonic phosphate buffer (310 mosM, pH 7.4) containing 0.1 mM EDTA. The supernatant and buffy coat were carefully removed after each washing.

Ghost preparation

Human erythrocyte ghosts were prepared by lysing packed cells in a 6-fold volume of a hypotonic phosphate buffer (20 mosM, pH 7.4) containing 0.1 mM EDTA, essentially as described by Dodge et al. [17]. After 45 min, the ghosts were centrifuged at $30000 \times g$, 0 °C for 20 min.

The ghosts were subsequently washed with the same volume of hypotonic buffer and again centrifuged. The washing procedure was repeated as many times as necessary to obtain the desired preparation (see Results). Ghosts were stored at −20 °C and used within one week. Aliquots of the ghost preparations were taken for lipid extraction and subsequent phosphorus determination.

Hemoglobin determination

Total hemoglobin in each ghost preparation was determined by the cyanmethemoglobin method [18]. To a suitable aliquot of ghost (usually 100 μl), 1 ml of Drabkin's reagent was added. 100 μl 5 mM SDS was added in order to yield a clear solution. Absorbance was read at 540 nm.

The above hemoglobin determination appeared to be not sensitive enough to clearly discriminate hemoglobin levels in ghost preparations after 4, 5 and 6 washes. We therefore developed an alternative procedure to measure low concentrations of hemoglobin in erythrocyte ghosts. This procedure is based on our observation that hemoglobin present in the ghost preparations quenches the fluorescence signal of added *cis*-parinaric acid in a concentration dependent way. The extent of quenching of the fluorescence signal was calibrated using known amounts of hemoglobin. Referring to the obtained standard quench curve, hemoglobin concentration in a ghost membrane sample can be calculated from the height of the fluorescence signal after addition of the *cis*-parinaric acid, provided that sample and standard curve are determined under identical conditions.

Lipid analysis

Extraction of lipids was performed as described by Rose and Oklander [19].

The phospholipid content of membranes was determined as inorganic phosphate according to Rouser et al. [20].

For gaschromatographic determination of the fatty acid composition of membrane samples, a direct transesterification method was employed, without a prior lipid extraction procedure. To a sample equivalent to 100 μl of packed cells in a screw capped tube was added: 100 μl of butylated hydroxytoluene (10 mg/ml MeOH) as antioxidant, and 25 μl of heptadecanoic acid ($C_{17:0}$, 2 mg/ml MeOH) as internal standard. In order to transesterify the fatty acyl chains into the corresponding methyl esters, 2 ml of 1 M methanolic HCl was added. The tube was sealed after replacing the air by nitrogen and then heated for 2 h at 90 °C. The methyl esters were extracted into 2 ml of hexane. This extraction was repeated twice. The combined hexane extracts were evaporated under a stream of nitrogen. The methyl esters were taken up again in 1 ml of hexane, a 1 μl aliquot of which was injected into the gaschromatograph. The instrument used was a Shimadzu GC 9A capillary gas chromatograph equipped with a 30 m, 0.25 mm i.d. SP 2330 column (Supelco, Bellefonte, PA, U.S.A.). The temperatures of the splitless injector and F.I.D. were maintained at 270 °C. The initial oven temperature of 100 °C was maintained for 1 min. The column was then heated up to 170 °C at 20 C°/min, kept at 170 °C for 5 min, then heated again up to 240 °C at 10 C°/min, and finally maintained at 240 °C for 8.5 min. The production of thiobarbituric acid reactive substances was measured according to Bidlack and Tappel [21].

Fluorescence measurements

Fluorescence measurements were carried out in a thermostated cuvet equipped with a magnetic stirring device using a Perkin-Elmer MPF-3 Fluorescence Spectrophotometer. The excitation wavelength used in the parinaric acid assay was 324 nm and the emission wavelength 413 nm.

The buffer used contained 10 mM Tris, 150 mM NaCl (pH 7.4) (referred to as buffer throughout). *cis*-Parinaric acid was added to a ghost suspension by injection of a small volume of an ethanolic solution. The fluorescence intensity of *cis*-parinaric acid was recorded in time. The slow rate of parinaric acid fluorescence decrease with no oxidants added was taken as baseline. The rate of fluorescence decrease after addition of oxidants was determined and taken as a measure for the rate of peroxidative degradation of the probe molecules.

Unless indicated otherwise, experiments were carried out at 25 °C.

Results

The standard quench curve obtained from addition of hemoglobin to a white ghost suspension containing *cis*-parinaric acid is shown in Fig. 1. At the hemoglobin concentrations indicated, a linear relationship exists between hemoglobin concentration and the logarithm of *cis*-parinaric acid fluorescence intensity (expressed as a percentage of the unquenched signal). At higher concentrations, deviation from linearity occurs.

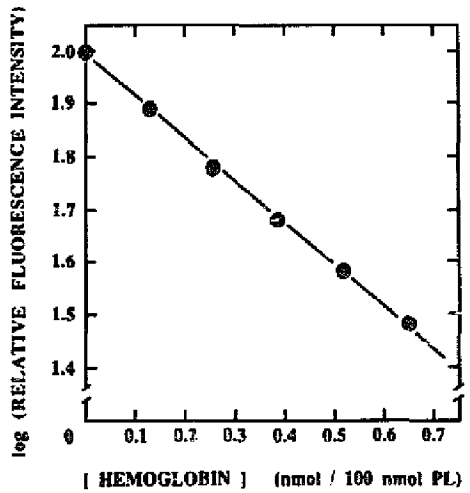

Fig. 1. Standard quench curve for the fluorimetric determination of the hemoglobin content of erythrocyte ghost membrane suspensions. 1.5 μM *cis*-parinaric acid was added to a white ghost suspension in buffer (200 μM of phospholipid), containing virtually no residual hemoglobin. Fluorescence intensity of *cis*-parinaric acid was recorded at 324 nm excitation and 413 nm emission. Known amounts of hemoglobin were added and the quenching of parinaric acid fluorescence was followed as a function of hemoglobin concentration. Recorded fluorescence intensities were corrected for a small baseline signal from membranes alone, fluorescence intensity without addition of hemoglobin was set at 100%. Hemoglobin concentrations are given in nmol hemoglobin per 100 nmol phospholipid in the assay system. To obtain μM equivalents, a given concentration should be multiplied by a factor 2. To obtain μM concentrations on a heme basis, a given hemoglobin concentration should be multiplied by a factor 8.

Experimental conditions in the parinaric acid assay have to be chosen such, that fluorescence intensity of the probe is a linear function of its concentration. Also, the membrane concentration should be sufficiently high to eliminate any significant contribution of probe molecules still present in the water-phase. Concentration curves defining the relationship between fluorescence intensity and concentration of *cis*-parinaric acid in human erythrocyte ghost membranes are presented in Fig. 2.

In Fig. 2A, fluorescence intensity is shown to increase upon addition of *cis*-parinaric acid. This indicates incorporation of the probe molecules into the membranes, as fluorescence of parinaric acid in micellar structures that are formed after injection of an ethanolic solution in water is completely self-quenched. It is clear that in pink, hemoglobin-rich, ghosts parinaric acid fluorescence is strongly quenched as compared to white, hemoglobin-poor, ghosts.

Addition of white ghosts to buffer containing parinaric acid in micellar configurations increases the fluorescence intensity as the self-quenching of the parinaric acid molecules is diminished by 'dissolving' of the parinaric acid molecules in the membrane (Fig. 2B). Addition of the pink ghosts also lessens self quenching of parinaric acid, thus increasing the fluorescence signal. In this case,

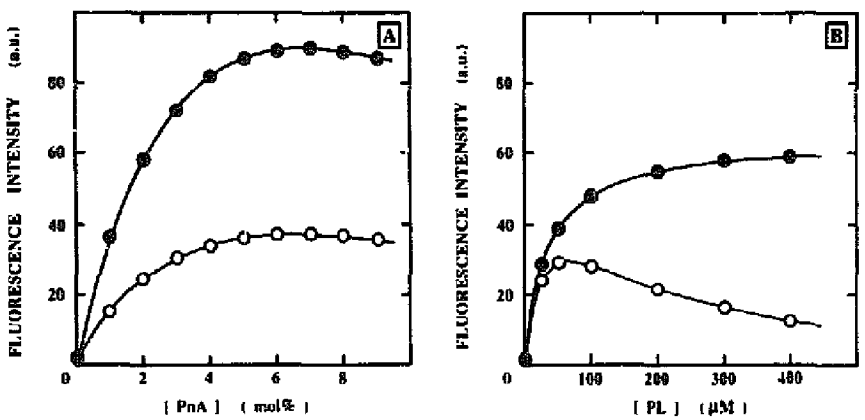

Fig. 2. Fluorescence intensity of *cis*-parinaric acid in human erythrocyte ghost membranes as a function of concentration. Curves are shown for (○) pink ghosts, residual hemoglobin concentration 0.55 nmol/100 nmol phospholipid, and (●) white ghosts, residual hemoglobin concentration 0.01 nmol/100 nmol phospholipid. (A) 2 ml of buffer containing ghost membranes equivalent to 200 μM phospholipid and varying concentrations of *cis*-parinaric acid, added by injection from an ethanolic solution. Parinaric acid concentration is expressed as mol% relative to phospholipid. (B) 2 ml of buffer containing 1.5 μM *cis*-parinaric acid and various amounts of ghost membranes, expressed as μM equivalents of phospholipid.

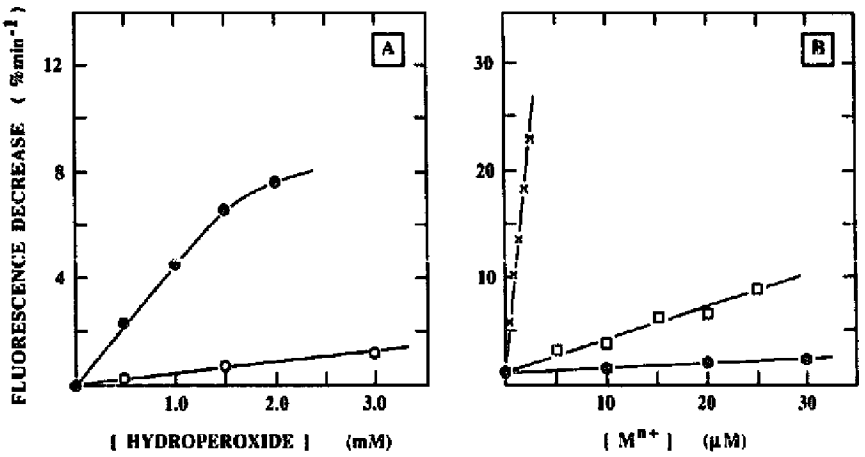

Fig. 3. Peroxidative degradation of *cis*-parinaric acid in human erythrocyte white ghost membranes (residual hemoglobin content 0.02 nmol/100 nmol phospholipid). Different peroxidation initiating systems were used. Parinaric acid was added to a ghost membrane suspension (200 μM phospholipid in a total volume of 2 ml of buffer) and fluorescence intensity was recorded in time. Upon addition of oxidants the rate of parinaric acid degradation was determined as the rate of fluorescence decrease. Hydroperoxides were added by injection from a concentrated ethanolic solution. (A) Peroxidation initiated by various concentrations of cumene hydroperoxide (●) or *tert*-butyl hydroperoxide (○) in the presence of 20 μM Cu^{2+}. (B) Influence of metal ion cofactors on cumene hydroperoxide induced peroxidation of *cis*-parinaric acid. Cumene hydroperoxide concentration was 1.5 mM in all measurements, metal ion concentrations are varied: Fe^{3+} (●), Cu^{2+} (□), hemin-Fe^{3+} (×).

however, there is an opposing effect as well, since addition of more ghost membranes causes an increase in hemoglobin concentration, resulting in increasing quenching of the fluorescence signal of parinaric acid. The combination of these two effects accounts for the shape of the curve for pink ghosts, as shown in Fig. 2B.

From these observations it was decided to use a ghost membrane concentration equivalent to at least 200 μM of phospholipid. As with vesicle membranes [7], a probe concentration of up to about 1 mol% gives a virtually linear relationship between probe concentration and fluorescence intensity.

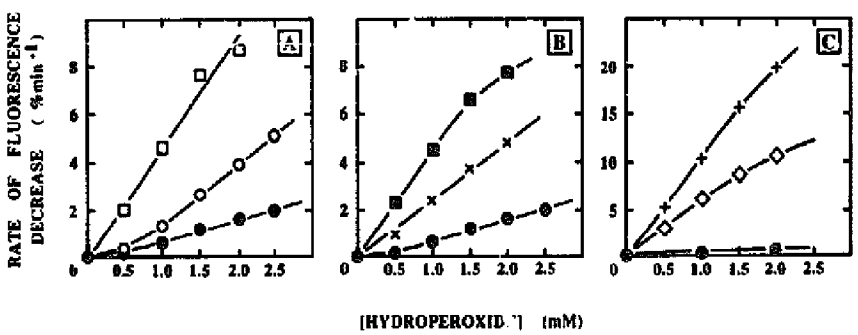

Fig. 4. Comparison of the effects of hemoglobin, Cu^{2+}, and hemin-Fe^{3+} on cumene hydroperoxide induced peroxidation of *cis*-parinaric acid in human erythrocyte ghosts. General conditions as in Fig. 3. (A) Varying concentrations of residual hemoglobin in the course of ghost preparation affect the rate of parinaric acid peroxidation. Ghost membranes containing 0.02 (●), 0.05 (○), 0.50 (□) nmol hemoglobin/100 nmol phospholipid. (B) Varying concentrations of Cu^{2+} added to a white ghost suspension (0.02 nmol hemoglobin/100 nmol phospholipid). No Cu^{2+} added (●), 10 μM Cu^{2+} (×), 20 μM Cu^{2+} (■). (C) Varying concentrations of hemin-Fe^{3+} added to a white ghost suspension (0.01 nmol hemoglobin/100 nmol phospholipid). No hemin-Fe^{3+} added (●), 0.25 μM hemin-Fe^{3+} (◇), 0.50 μM hemin-Fe^{3+} (+).

Fig. 3 illustrates that the rate of peroxidative degradation of parinaric acid in erythrocyte ghosts strongly depends on the combination of initiator and cofactor used. Fig. 3A clearly demonstrates that cumene hydroperoxide is a more powerful initiator as compared to *tert*-butyl hydroperoxide. At a fixed concentration of Cu^{2+}, the initial decrease in fluorescence intensity of *cis*-parinaric acid, indicative of its peroxidative degradation, is a linear function of the hydroperoxide concentration for both initiators used.

In addition to the type of initiator, the rate of peroxidation also depends on the cofactors present in the system. This is shown in Fig. 3B for Fe^{3+}, Cu^{2+}, and hemin-Fe^{3+} as cofactors. In combination with cumene hydroperoxide, hemin-Fe^{3+} appears to be the most powerful, followed by Cu^{2+}. Fe^{3+} is the least effective of the three.

Fig. 4 compares the effects of hemoglobin, Cu^{2+}, and hemin-Fe^{3+} on the peroxidation of *cis*-parinaric acid in ghosts as a function of cumene hydroperoxide concentration. Addition of cumene hydroperoxide alone to a white ghost suspension, containing very small amounts of residual hemoglobin, results in a significant degradation of *cis*-parinaric acid only at high concentrations of hydroperoxide. The presence of a transition metal ion cofactor in the ghost suspension, however, greatly enhances the effect of cumene hydroperoxide. This is demonstrated in Fig. 4A for residual hemoglobin present after 3, 4 and 5 washes, and in Figs. 4B and 4C for the addition of, respectively, Cu^{2+} and hemin-Fe^{3+} to white ghost suspensions.

To correlate parinaric acid degradation in pink and white ghosts with conventional techniques for detecting lipid peroxidation, pink and white ghost membrane preparations were incubated for 1 h at 37°C in the presence of cumene hydroperoxide/hemin-Fe^{3+}. Results are shown in Fig. 5. On incubation with cumene hydroperoxide/hemin Fe^{3+}, a higher production of thiobarbituric acid reactive substances is observed in pink ghosts when compared to white ghosts (Fig. 5A). These results are comparable to those shown in Fig. 4B using the parinaric acid method, again indicating a peroxidation promoting effect of hemoglobin. Peroxidative degradation of fatty acyl chains was examined by gaschromatographic analysis after

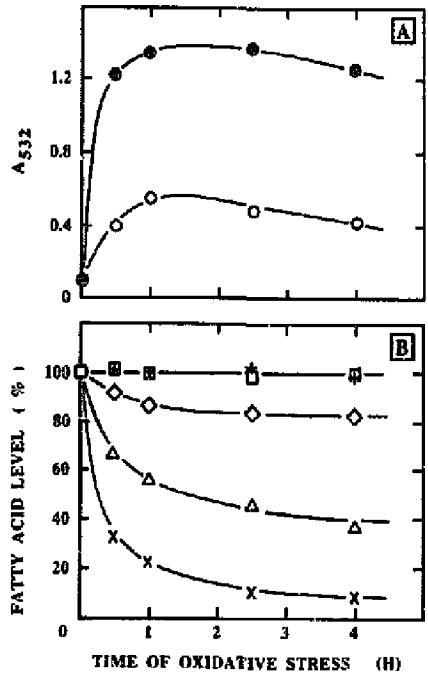

Fig. 5. Lipid peroxidation in human erythrocyte ghost membranes as monitored by the production of thiobarbituric acid-reactive substances and gaschromatography of fatty acid methylesters. Pink and white ghost membranes (hemoglobin concentration 0.12 and 0.02 nmol/100 nmol phospholipid, respectively) were incubated at 37°C at a membrane phospholipid concentration of 750 μM in the presence of 5 mM cumene hydroperoxide and 1 μM hemin. (A) Production of thiobarbituric acid-reactive substances, expressed as absorbance at 532 nm. Pink ghost (●), white ghost (○). (B) Fatty acid levels in pink ghosts of palmitic acid $C_{16:0}$ (□), stearic acid $C_{18:0}$ (+), oleic acid $C_{18:1}$ (◇), linoleic acid $C_{18:2}$ (△), and arachidonic acid $C_{20:4}$ (×), expressed relative to control value.

direct transesterification of membrane samples with HCl/MeOH as described in Materials and Methods. This method proved to yield reproducible and accurate results for intact erythrocytes as well as ghost membrane preparations. As an example, Fig. 5B shows the time course of fatty acid peroxidation of the five major fatty acids present upon incubation of a pink ghost membrane suspension with cumene hydroperoxide/hemin-Fe^{3+}. The rate of fatty acid degradation is dependent on the degree of unsaturation, as could be expected. This is also true for white ghosts, although the changes in fatty acid levels are much less pronounced (results not shown).

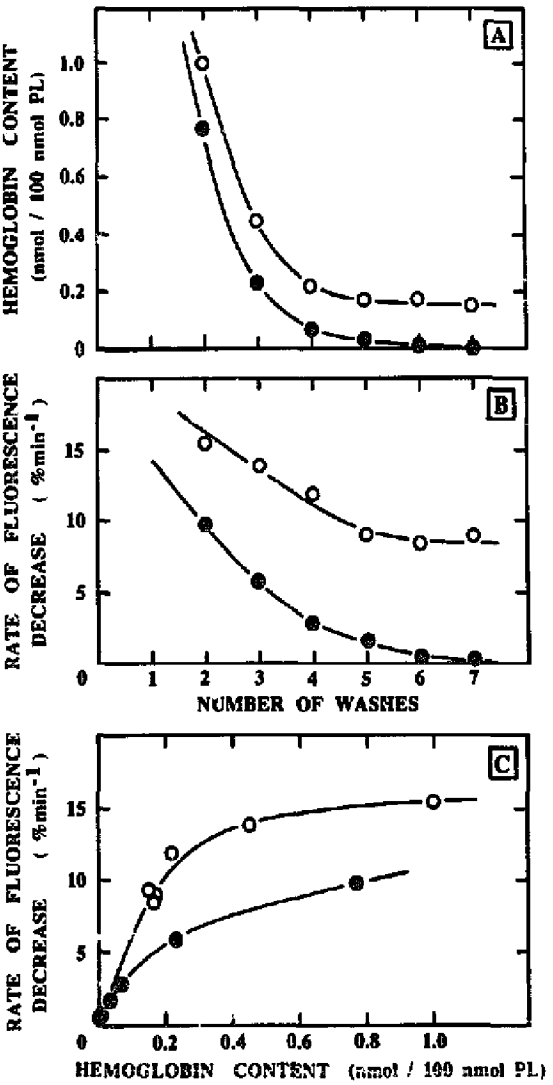

Fig. 6. Hemoglobin content and peroxidation of *cis*-parinaric acid in human erythrocyte ghost membranes at various time-points in preparation. The preparation procedure is described in Materials and Methods. The number of washes does not include the first addition of hypotonic buffer to cells in order to obtain the initial hemolysate. Normal (●), sickle (○) erythrocyte ghost membranes. (A) Residual hemoglobin content of human erythrocyte ghosts as a function of the number of washes. (B) Peroxidation of *cis*-parinaric acid in erythrocyte ghosts as a function of the number of washes. To ghost membranes (200 μM of ghost phospholipid in a total volume of 2 ml of buffer) *cis*-parinaric acid was added (1.5 μM). Fluorescence intensity was recorded in time. Upon addition of 1.5 mM cumene hydroperoxide, the relative rate of fluorescence decrease was calculated. Each point represents the average of three measurements. (C) Results from (A) and (B) in a plot of parinaric acid fluorescence decrease rate as a function of hemoglobin content of the ghosts.

The hemoglobin content of a ghost preparation decreases with the number of washes, as is shown in Fig. 6A for normal and sickle erythrocyte ghosts. Although the preparation procedure in both cases is identical, the sickle erythrocyte ghosts clearly contain more hemoglobin after each wash, indicative of an enhanced binding of hemoglobin to the membrane. Fig. 6B shows that the higher hemoglobin content of sickle erythrocyte ghosts as compared to normal erythrocyte ghosts is accompanied by a higher rate of *cis*-parinaric acid degradation after addition of cumene hydroperoxide.

When combining the data of Figs. 6A and 6B, the rate of fluorescence decrease of *cis*-parinaric acid can be plotted as a function of hemoglobin content of the ghost (Fig. 6C). It is evident that, even at equal concentrations of hemoglobin, the rate of fluorescence decrease of parinaric acid is much higher in sickle erythrocyte ghost membranes as compared to normal erythrocyte ghost membranes. To investigate if this difference could indeed be attributed to different properties of the hemoglobins, cumene hydroperoxide induced per-

TABLE I

THE INFLUENCE OF NORMAL AND SICKLE HEMOGLOBIN ON PEROXIDATION OF *cis*-PARINARIC ACID IN HUMAN ERYTHROCYTE GHOST MEMBRANES, INITIATED BY CUMENE HYDROPEROXIDE

Parinaric acid assay conditions as in Fig. 6. Values given are mean ± S.D. for four measurements.

Hb effect measured [a]	Type of Hb added	[Hb] (nmol/100 nmol phospholipid)	Rate of *F* decrease ($\%min^{-1}$)
Reference white ghost	–	0.015 ± 0.010	0.77 ± 0.16
Total Hb	HbA	0.105 ± 0.010	2.73 ± 0.06
Total Hb	HbS	0.105 ± 0.010	3.27 ± 0.17
Membrane Hb	HbA	0.030 ± 0.010	1.94 ± 0.06
Membrane Hb	HbS	0.035 ± 0.010	2.32 ± 0.18

[a] Total Hb: parinaric acid assay performed directly after addition of hemoglobin to white ghost membranes in buffer. Membrane Hb: same amount of hemoglobin was added as for total Hb measurement. Ghost was centrifuged 5 min after addition of hemoglobin. Ghost pellet was resuspended in buffer. Hemoglobin content and peroxidation of parinaric acid were subsequently measured.

oxidation of parinaric acid was measured in normal erythrocyte white ghosts to which either normal or sickle hemoglobin (as hemolysate) has been added. The results in Table I show a higher rate of fluorescence decrease in the presence of sickle hemoglobin than with equal amounts of normal hemoglobin. This applies for equal total hemoglobin concentrations as well as for equal concentrations of membrane-bound hemoglobin.

Discussion

There is a large body of evidence suggesting that oxygen radicals, lipid peroxidation, and peroxidation products are involved in many pathological conditions of man [3,22]. The erythrocyte is often used as a model system in peroxidation studies, as this membrane is easily obtained and purified. Furthermore, the erythrocyte is a typical example of a cell under constant oxidative stress, inherent to its function as oxygen transporter. This oxidative stress may come from external sources as a consequence of contact with metabolically active cells [22,23]. It may also be generated internally, for example, by the release of superoxide radical (O_2^-) from a hemoglobin-oxygen complex [24,25]. In order to protect the RBC membrane against deleterious effects of oxygen radicals a complex protective system is present [8]. This protective system is very efficient under normal conditions, and oxidative damage will therefore only occur if these defenses are overcome.

In the present study, the use of the parinaric acid assay for peroxidation studies with erythrocyte ghost suspensions is described. Although erythrocyte ghosts no longer contain the complete antioxidant system present in intact erythrocytes, they appear to be very useful for studying erythrocyte membrane lipid peroxidation initiated by externally added oxidants.

Using the parinaric acid assay to investigate the effect of different peroxidation initiating systems in RBC membrane suspensions, clear differences in peroxidation rates were observed. An important factor in determining the rate of peroxidation appears to be whether damaging radicals are generated at the membrane level or in the water phase. Higher membrane concentrations of cumene (per)oxyl radicals may account for the observed difference in initiator efficiency between cumene hydroperoxide and *tert*-butyl hydroperoxide, the latter being more water soluble (Fig. 3A). The very high efficiency of hemin-Fe^{3+} as a cofactor compared to free Fe^{3+} (Fig. 3B) may in part be explained by the special properties of the Fe^{3+} complexes within the heme moiety, but the fact that hemin is membrane-bound to a large extent, whereas free Fe^{3+} is present in the water phase, will also contribute significantly to this observation.

Special attention should be drawn to the role of hemoglobin in the peroxidation process. Hemoglobin may give rise to oxygen radical formation, and may also act as a catalyst contributing to a continuous oxidative stress on the erythrocyte membrane. In general, our results obtained with the parinaric acid assay, as well as with conventional methods, support the idea of hemoglobin playing a stimulating role in the peroxidation process. It is shown that the presence of hemoglobin in erythrocyte membrane suspensions incubated with cumene hydroperoxide, strongly increases the rate of peroxidation of parinaric acid. The very small decrease rates observed in white ghost suspensions, incubated with cumene hydroperoxide, may very well be a result of the presence of minute amounts of residual hemoglobin. Comparison of the results for hemoglobin, Cu^{2+}, and hemin-Fe^{3+} (Fig. 4) suggests the involvement of the heme iron in catalysing radical formation from cumene hydroperoxide, although it is assumed that hemoglobin doesn't act as a true Fenton catalyst [2].

Radicals will be most effective at initiating lipid peroxidation if they are generated in or near the target membrane, and in this respect the role of membrane-bound hemoglobin could be very important. It is a well-known characteristic of sickle cells that they contain a larger amount of membrane-bound hemoglobin when compared to normal cells [26,27] and that this may be an important cause of the higher susceptibility towards oxidative stress, noted in sickle erythrocytes [10,28]. Higher concentrations of membrane-bound hemoglobin degradation products such as hemichrome and hemin [29,30] may also contribute significantly to the oxidative stress on the membrane [31]. In addition, it has been found that

sickle hemoglobin is more susceptible to autoxidation and generates more toxic oxygen species than normal hemoglobin [32].

Our results clearly confirm the above observations. With the preparation procedure used, it was impossible to obtain hemoglobin-free ghosts from sickle erythrocytes. Monitoring the ghost washing procedure with the parinaric acid assay, it appeared that the higher hemoglobin content of sickle erythrocyte ghost suspensions is accompanied by a higher peroxidation rate of parinaric acid (Fig. 6B). From Fig. 6C it is evident that even if ghost suspensions contain equal concentrations of, respectively, normal and sickle hemoglobin, the peroxidation rate of parinaric acid is much higher with sickle hemoglobin. Although this strongly suggests different peroxidation promoting features of sickle hemoglobin, these experiments do not exclude the possibility that other factors, like altered membrane characteristics and lowered vitamin E content, contribute to this observation. However, in experiments where hemoglobin was added to normal erythrocyte white ghost suspensions and peroxidation of parinaric acid subsequently monitored after addition of cumene hydroperoxide, similar differences in peroxidation promoting capabilities between normal and sickle hemoglobin were observed (Table I). These results indicate that peroxidation is indeed more effectively promoted by sickle hemoglobin than it is by normal hemoglobin. The exact cause of the observed difference cannot be deduced from these experiments. A possible explanation could be a different binding of sickle hemoglobin to the membrane, perhaps more deeply penetrating into the membrane surface. Alternatively, the configuration of the sickle hemoglobin molecule could be different, facilitating an enhanced reactivity towards cumene hydroperoxide.

Comparing hemoglobin behaviour in normal and sickle erythrocyte ghosts, it should furthermore be noted that also normal hemoglobin binds to the membrane, albeit to a lesser extent [33–35]. Shaklai et al. [34] calculated that there are a number of $1.2 \cdot 10^6$ high affinity binding sites for hemoglobin per cell in normal erythrocytes. In our situation, this would imply that already from the third ghost washing on there is an important role of membrane-bound hemoglobin in determining the outcome of the peroxidation experiments (Fig. 6).

The importance of membrane-bound hemoglobin in lipid peroxidation is also obvious from the results shown in Table I. From these data it can be calculated that membrane-bound hemoglobin is about 6-times more effective in promoting peroxidation than hemoglobin present in the water phase, both for normal and for sickle hemoglobin.

It was beyond the scope of this study to include a thorough analysis of the amounts of hemoglobin derivatives that might be present in our experiments. However, in the above experiments, hemoglobin was added to the ghosts as a fresh erythrocyte hemolysate, obtained after centrifugation to pellet the membranes. It can, therefore, be assumed that the hemolysate added to the ghosts contained no hemin or other membrane-bound hemoglobin denaturation products. Consequently, the effects described in Table I are indeed effects of hemoglobin.

Comparing the effect of hemoglobin to that of hemin under similar conditions (Fig. 4C), it appears that hemin is much more effective in promoting peroxidation (calculated on a heme basis) than either normal or sickle hemoglobin. The order of peroxidation promoting capabilities that can be derived from our experiments is: hemin > sickle hemoglobin > normal hemoglobin. This supports the generally accepted idea that hemin and other hemoglobin degradation products accumulating during the aging process, could contribute to an increased oxidative stress on the membranes of aged erythrocytes.

Some comments should be made regarding hemoglobin as a potential source of interference when present in the parinaric acid assay system. Interference from hemoglobin arises from its absorptive properties in the wavelength regions used in the assay. In pink erythrocyte ghost suspensions, the residual hemoglobin may cause a substantial quenching of the parinaric acid fluorescence signal (Fig. 2), but usually a suitable signal can be obtained.

Oxidation of hemoglobin will change its absorptive properties, which could influence the extent of quenching of the parinaric acid fluorescence signal. This means that oxidation of hemo-

globin in itself can be sufficient to cause a change in the recorded fluorescence signal, without parinaric acid necessarily being peroxidised. Thus, it could be difficult to correctly interpret the results from the parinaric acid assay. From absorption studies that were carried out (results not shown), it followed that the qualitative conclusions drawn from our peroxidation experiments with hemoglobin-containing ghosts are not influenced by this effect. Nevertheless, one has to be aware of the fact that the decrease in fluorescence recorded in these experiments may not be strictly proportional to the degradation rate of parinaric acid, as this parameter will also be affected by the oxidation of hemoglobin.

The effects of potentially interfering compounds as described here for hemoglobin should be studied in each system to which the parinaric acid assay is applied.

Summarising, the parinaric acid assay system has been shown to offer possibilities for studying peroxidation processes in biological membrane systems. Taking the human erythrocyte membrane as a model, several initiating systems were investigated, as well as the effect of residual hemoglobin in ghost membrane preparations. The effectivity of a radical generating system appeared to be strongly dependent upon whether radicals are generated at the membrane level or in the water phase. Sickle erythrocyte ghost membranes were shown to contain more hemoglobin as compared to normal erythrocyte ghost membranes, and this seemed to be an important factor in determining their higher susceptibility to peroxidation. In addition, it was shown that sickle hemoglobin characteristically promotes peroxidation more effectively than normal hemoglobin.

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