PERMEATION OF LIPOSOME MEMBRANE BY SUPEROXIDE RADICAL

G. V. RUMYANTSEVA, L. M. WEINER*, Yu. N. MOLIN and V. G. BUDKER+

Institute of Chemical Kinetics and Combustion, Novosibirsk, USSR and *+Institute of Organic Chemistry, Novosibirsk, USSR*

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1. Introduction

At present the role of the superoxide radical ($O_2^{\cdot-}$) in oxidation processes in the cell is under active investigation [1]. Of special importance is its function in reactions of microsomal oxidation [2,3] and in pathological processes proceeding in membranes [4].

Recently it has been reported that $O_2^{\cdot-}$ can cross the membrane of the erythrocyte, and that superoxide dismutase can inhibit the lysis of erythrocyte ghosts containing [^{14}C]sucrose [5]. It appears that $O_2^{\cdot-}$ can traverse the membrane of the erythrocyte through the anion 'channels' [6].

The objective of this work was to study the possibility of permeation of the model membrane of phosphatidylcholine liposomes by superoxide radical. By using liposomes containing potassium ferricyanide and superoxide dismutase we have shown that enzymically produced $O_2^{\cdot-}$ can cross a lipid bilayer membrane and penetrate inward. The penetration of $O_2^{\cdot-}$ is affected by the structural state of the membrane.

2. Materials and methods

Xanthine (Sigma) and dipalmitoylphosphatidylcholine (Serva) were used for experiments. The activity of xanthine oxidase (Sigma) was determined from the variation of absorbance (λ = 295 nm) during xanthine–urate transformation in 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 0.5 mM xanthine. The enzyme was used at 1.3×10^{-8} M to oxidize xanthine at a rate of 2×10^{-5} mol/l . min. Superoxide dismutase (SOD) from ox blood was kindly given by R. Nalbandyan (Institute of Biochemistry, Erevan). The addition of 2×10^{-7} M SOD inhibited oxidation of nitroblue tetrazolium (NBT) by a factor of 15 in the system: NADH–phenazine methosulfate (PMS) (6×10^{-6} M NADH; 10^{-5} M PMS). Phosphatidylcholine was isolated from eggs and purified on Al_2O_3 as in [7]. It was additionally purified on silica gel as in [8]. Two-dimensional thin-layer chromatography on silica gel was employed to characterize lipid. The medium for the first dimension was chloroform:methanol:H_2O (65:25:4), that for the second dimension chloroform:methanol:concentrated NH_3 (14:6:1).

Liposomes were prepared as in [9] in the buffer I (50 mM KH_2PO_4, 0.1 M EDTA, (pH 7.8)) with ferricyanide (0.1 M) being in the internal volume. Residual ferricyanide was removed on a Sephadex G-25 column.

Liposomes containing SOD were prepared as in [10] and residual enzyme was removed on Sepharose 4B. The activity of SOD sealed into liposomes was tested by way of NADH–PMS–NBT reaction. Ferricyanide was reduced in the buffer I containing 0.7 mM xanthine and 1.3×10^{-8} M xanthine oxidase. The reaction was examined by using the differential method (decrease of A_{420}). The rate of the ferricyanide reduction decreases with oxygen consumption in the reaction mixture. Therefore, the reaction rate was determined from the initial regions of the kinetic curves. The reaction product, $K_4Fe(CN)_6$, was detected in liposomes by the following procedure. Gel-filtration on Sephadex G-25 was followed by extraction of lipid from the liposome fraction by chloroform–methanol (1:1). Residual chloroform

* To whom correspondence should be addressed

was removed from the water–methanol mixture by washing with hexane 4 times. The solution was evaporated and dissolved in the distillate prior to registration of ultraviolet spectra. An Aminko DW2 spectrophotometer was used.

3. Results

The rate of reduction of freely dissolved ferricyanide by the xanthine oxidase system generating $O_2^{\cdot-}$ significantly exceeds that of reduction of liposome-entrapped ferricyanide (cf. table 1). The addition of Triton X-100 (0.5%) accelerated the reaction in liposomes. The observed slowdown of the reduction rate (table 1) is not associated with the chemical interaction of $O_2^{\cdot-}$ with the membrane components. In fact:

(i) Addition of 'empty' liposomes did not affect the reaction of $O_2^{\cdot-}$ with $K_3Fe(CN)_6$ in the solution;
(ii) The A_{233}/A_{215} ratio, which indicates the appearance of the lipid peroxide oxidation products [11], was constant before and after the experiments with liposomes containing $K_3Fe(CN)_6$;
(iii) The resulting lipid was found to be identical to the initial one as shown by thin-layer chromatography on silica gel (see section 2).

The observed kinetic behaviour of $K_3Fe(CN)_6$ reduction is independent of the possible release of ferricyanide from liposomes. This is consistent with the fact that the reaction product, $K_4Fe(CN)_6$, could be detected in the liposome fraction after that had been subjected to another gel-filtration step at the end of the experiment (fig.1).

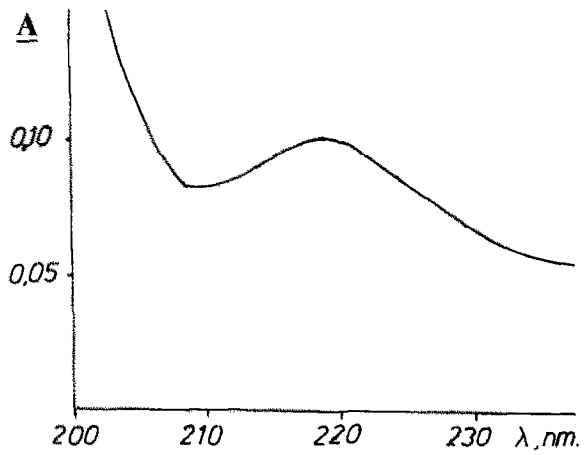

Fig.1. Absorption spectrum of reaction product, $K_4Fe(CN)_6$, isolated from liposomes (see section 2). $K_3Fe(CN)_6$ (7×10^{-5} M) was reduced and $K_4Fe(CN)_6$ (3.6×10^{-5} M) was isolated.

Liposomes containing superoxide dismutase were used to prove penetration of the superoxide radical through the membrane. These liposomes inhibit the reduction of $K_3Fe(CN)_6$ as detailed in fig.2.

In order to ascertain whether the permeation of the membrane by $O_2^{\cdot-}$ is dependent on the nature of the lipid and its structural state, we used liposomes made from synthetic dipalmitoylphosphatidylcholine whose phase transition occurs at 41°C. In this case, $O_2^{\cdot-}$ interacts with ferricyanide inside liposomes only at temperatures above that of the lipid phase transition (fig.3).

The reduction of both free and liposome-entrapped

Table 1
Comparison of reduction rates of $K_3Fe(CN)_6$ in solution and in liposomes in the presence of xanthine-xanthine oxidase

Reaction in solution		Reaction in liposomes	
Initial $[K_3Fe(CN)_6]$ (M)	Rate (mol/l. s)	Initial $[K_3Fe(CN)_6]$ (M)	Rate (mol/l. s)
3.8×10^{-4}	5.0×10^{-7}	3.0×10^{-4}	1.4×10^{-8}
2.7×10^{-4}	4.1×10^{-7}	2.6×10^{-4}	1.2×10^{-8}
4.0×10^{-4}	4.7×10^{-7}	4.0×10^{-4}	7.7×10^{-9}
3.1×10^{-4}	4.7×10^{-7}	3.0×10^{-4}	8.3×10^{-9}
2.75×10^{-4}	4.3×10^{-7}	3.0×10^{-4}	1.3×10^{-8}

Results of 5 different experiments; Lipid, 5×10^{-3} M

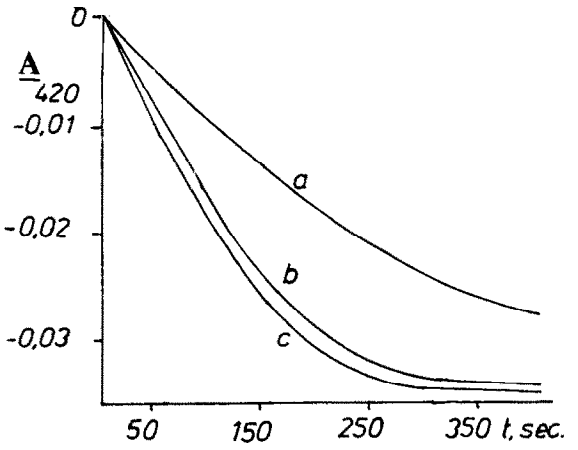

Fig.2. Reduction of $K_3Fe(CN)_6$: (a) in liposomes containing SOD; (b) in the presence of 'empty' liposomes; (c) control reaction in the presence of freely dissolved ferricyanide. $K_3Fe(CN)_6$, 2.5×10^{-5} M; Lipid, 10^{-3} M; SOD, 10^{-8} M.

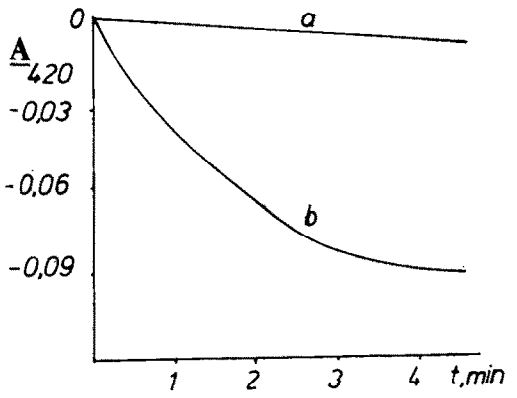

Fig.3. Reduction of $K_3Fe(CN)_6$ sealed into liposomes composed of synthetic lipid by the xanthine oxidase system. (a) $T = 25°C$; (b) $T = 45°C$. $K_3Fe(CN)_6$, 1.6×10^{-4} M; Lipid, 5×10^{-3} M.

ferricyanide is accelerated ~3 fold as the reaction mixture is increased from pH 7.8–9 (cf. table 2).

4. Discussion

The results presented here suggest that the superoxide radical can penetrate into the inner liposome volume through the bilayer lipid membrane. This is supported by:

(i) The ferricyanide–ferrocyanide transformation in the liposomes caused by the interaction with $O_2^{\cdot-}$ generated outside (table 1, fig.1);

(ii) Inhibitory effect of liposomes containing superoxide dismutase on the formation of ferrocyanide in the solution (fig.2).

The fact that reduction of $K_3Fe(CN)_6$ proceeds more slowly inside liposomes than in the solution (table 1) may be related to the restrictions imposed by $O_2^{\cdot-}$ diffusion through the membrane. In fact, the decomposition of the membrane by detergent, Triton X-100, accelerates the reaction. It is known that solubility of anions and their diffusion coefficients are lower in membranes than in water [12]. Therefore, the state of the membrane should affect the reaction rate which is consistent with our observations in the case of synthetic lipid when the reaction occurs only at temperatures exceeding that of the lipid phase transition (fig.3). This was also the case for the interaction of $O_2^{\cdot-}$ with ferricyanide localized in liposomes from egg lecithin observed by us at 25°C (i.e., at temperatures above that of the phase transition). It should be noted that penetration of another activated oxygen form, namely singlet oxygen ($^1O_2$),

Table 2
Effect of pH on the rate of $K_3Fe(CN)_6$ reduction in solution and in liposomes

pH	Reaction in solution		Reaction in liposomes	
	Initial $[K_3Fe(CN)_6]$ (M)	Rate (mol/l · s)	Initial $[K_3Fe(CN)_6]$ (M)	Rate (mol/l . s)
7.8	4.0×10^{-4}	5.0×10^{-7}	4.0×10^{-4}	7.0×10^{-9}
9	4.3×10^{-4}	1.5×10^{-6}	3.4×10^{-4}	2.1×10^{-8}

Lipid, 5×10^{-3} M

into liposomes also depends upon the structural state of the membrane. The penetration is enhanced when the temperature is above the phase transition temperature [13].

The scatter in the values of the reduction rates of ferricyanide localized in liposomes (table 1) may be explained by $O_2^{\cdot-}$ diffusion through the membrane of liposomes with different multilayer composition.

Acceleration of $K_3Fe(CN)_6$ reduction in liposomes with increasing the reaction mixture from pH 7.8–9 (cf. table 2) implies that the $HO_2^{\cdot}$ radical does not cross the membrane. The concentration of this radical tends to decrease as the pH is increased. That such acceleration occurs may be ascribed to the slowdown of one of the most probable competitive reactions for $O_2^{\cdot-}$:

$$O_2^{\cdot-} + HO_2^{\cdot} \xrightarrow[k]{H^+} H_2O_2 + O_2 \text{ where } k \sim 10^8 \text{ mol/l . s} \quad [14]$$

This effect may be caused by the fall of $HO_2^{\cdot}$ concentration. The quantitative estimation of the effect of pH on the rate of $K_3Fe(CN)_6$ reduction with $O_2^{\cdot-}$ should take into account the activity of xanthine oxidase (which may increase with pH [15]).

Thus, our data indicate that the $O_2^{\cdot-}$ radical can cross the bilayer lipid membrane without special carriers or channels [6]. For this penetration, the structural state of the membrane is very important and may somehow regulate the biological processes involving $O_2^{\cdot-}$ radicals in the cell.

Acknowledgement

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