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THE EFFECTS OF GLUTARALDEHYDE AND OSMIUM TETROXIDE ON THE ERYTHROCYTE MEMBRANE

A SPIN LABEL STUDY

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A nitroxide spin label probe technique was applied to study the interaction between glutaraldehyde or osmium tetroxide (OsO_4) and the membranes of horse erythrocytes, ghosts and liposomes prepared from erythrocyte lipids. Two major conclusions have been established: (1) Reaction of the fixation reagents with the membrane is selective. OsO_4 reacts predominantly with lipids and glutaraldehyde with membrane proteins. (2) The lipid-protein interactions change after pretreatment by OsO_4 or glutaraldehyde.

Introduction

Osmium tetroxide (OsO_4) and glutaraldehyde have been widely used as fixating agents in electron microscopic studies of biological samples [1]. The mechanism of interaction of these reagents with various cellular components has been recently discussed by many authors [2–14]. It was found that OsO_4 and glutaraldehyde in millimolar concentration could be explored as specific reagents to modify biological membranes of intact erythrocytes [15,16]. As has been reported, erythrocyte membranes treated by OsO_4 reveal the products of interaction of OsO_4 with unsaturated lipids. These are cyclic esters of Os(VI) containing a *cis*-diol linkage [2–10]. Such cyclic monoesters may be formed as diesters [6], or dimeric monoesters [3] between two adjacent unsaturated lipids. The effect of glutaraldehyde treatment on the erythrocyte membrane has also been extensively studied [9,13,14]. This agent interacts with membrane proteins to form crosslinks with the proteins. Less is known about its action on the lipid portion of the membrane and the information is contradictory. Jost et al. [17] have found no effect of 2% soln. either on the fatty acids in lobster nerves or in the phospholipid liposomes. Effects were however observed by Shiga et al. [18].

In the present study the spin label method has been used to estimate the specific character of both agents and the changes they produce in the membrane structure.

Materials and Methods

Erythrocytes. Fresh, heparinized horse blood was used. It was centrifuged for 10 min at $1000 \times g$, plasma and leukocytes were removed. Erythrocytes were washed four times with an isotonic phosphate buffered NaCl solution pH = 7.4 and suspended in this solution.

Lipid extract and liposomes preparation. The lipids were extracted from erythrocyte ghosts with

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cold *n*-butanol at 0°C [19]. They were dried under vacuum in a glass tube and suspended in 25 mM buffer Tris-HCl, pH 7.4. This mixture was mechanically shaken to the final milky suspension. The concentration of lipids in the buffer was 50 mg/ml.

Preparation of erythrocyte ghosts. Erythrocyte ghosts were prepared by a standard technique of gentle hemolysis in a hypotonic solution of phosphate buffer at 4°C according to Dodge et al. [21].

The spin-labeled preparates. The spin probes 12-doxylstearate methyl ester, I(5,10), and 5-doxylpalmitate methyl ester, I(10,3), dissolve in the lipid bilayer. The spin labels *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) iodoacetamide (ISL) and *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (MSL) bind to proteins.

An appropriate amount of $2 \cdot 10^{-3}$ M ethanolic solution of I(m, n) was used to maintain the label to lipid molecular ratio 1:50. Then, the above solutions were dried on the tube walls and the subsequent suspensions mechanically shaken with erythrocytes, ghosts or liposomes. After 20 min the membranes were sufficiently labeled for the ESR experiment.

The packed ghosts were incubated overnight with an excess of MSL or ISL at 4°C. After the incubation period the unbound label was removed by washing six times with a phosphate buffer, pH 7.4.

Modification of erythrocytes, ghosts and liposomes. The erythrocyte ghost pellet was diluted in a phosphate buffer, pH 7.4, and various amounts of the modifying agent were added and incubated at 20°C for 1 h. Subsequently, the erythrocytes were washed three times by the phosphate buffer to remove the excess of the modifying agent.

The appropriate concentrations of agents were added into the suspension of liposomes in Tris-HCl buffer and the solution was shaken extensively. The volume ratio of the liposomes mixture to the modifying solution was constant.

All chemicals employed were of the reagent grade purity. Redistilled water was used.

Electron spin resonance spectroscopy. All spectra were recorded on the SE/X-28 Spectrometer manufactured at the Technical University of Wrocław. In order to estimate semiquantitatively the mobility of the spin probe, an empirical motion parameter R was calculated according to the following formula [22,23]:

$$R = W_0\left\{\left(\frac{h_0}{h_{-1}}\right)^{-1/2} - 1\right\}$$

where W_0, h_0, h_{-1} are defined in Fig. 1. An increase in the rate of molecular motion of the probe is characterized by a decrease in the value R. For MSL and ISL another parameter H was estimated which was equal to the ratio h_{II}/h_I [24], where h_{II} and h_I are also defined in Fig. 1. Such a procedure is applicable in this case because the ESR spectra of both spin labels were obtained with evidently split peak h_{+1}: two components of this band were distinguished, i.e. a strongly h_I and weakly h_{II} immobilized spin label.

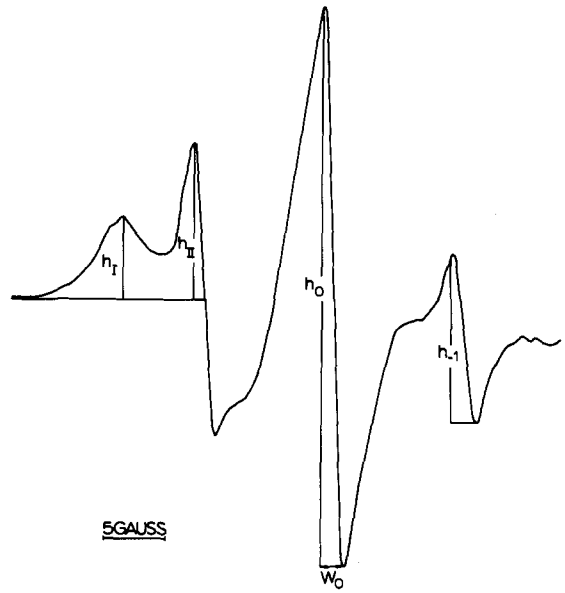


Fig. 1. The representative spectrum of MSL spin label bound to erythrocyte membrane. On the figure are defined variables whose functions are parameters R and H.

Results

Figs. 2–4 illustrate the effects of OsO_4 and glutaraldehyde concentration on the mobility of the spin label incorporated into the liposomes of intact erythrocytes or ghost membranes. Fig. 2A, curves c and d present the effects of OsO_4 on liposomes. The motion of spin label I(10, 3) is

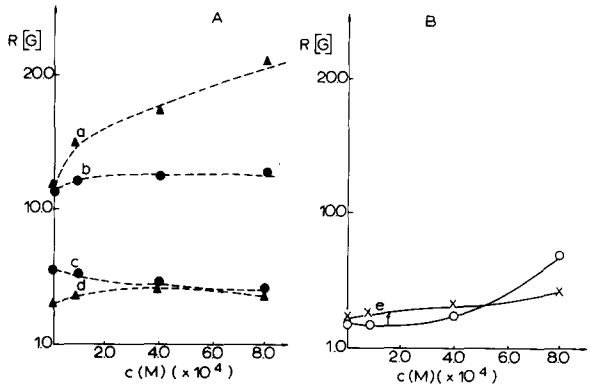


Fig. 2. (A) The effects of OsO_4 concentration on the mobility of the I(5, 10) (●— — —●) and I(10, 3) (▲— — —▲) spin probes incorporated into: a and b, intact erythrocytes; c and d, liposomes from erythrocyte lipids. (B) The effects of OsO_4 concentration on the mobility of the MSL (e) and ISL (f) spin labels incorporated into ghosts.

observed near the polar heads in the lipid bilayer. It has been found to decrease with the increased concentration of OsO_4. However, down in the hydrocarbon chains, weak immobilization of the label I(5, 10) motion is observed (Fig. 2A, curve c). Glutaraldehyde does not change the motion of the nitroxide group I(10, 3) (Fig. 3A, curve d), but rather slightly immobilizes I(5, 10) (Fig. 3A, curve c). These differences of effects that result

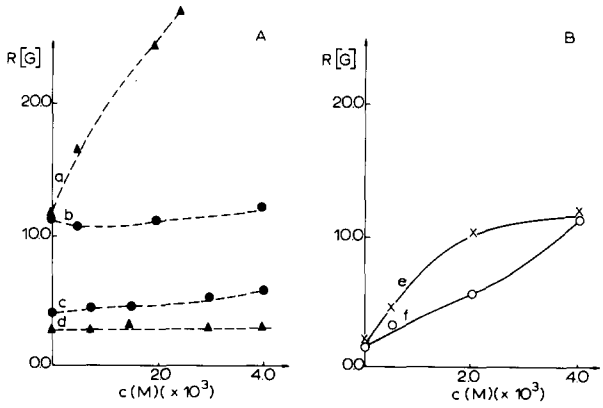


Fig. 3. (A) The effects of glutaraldehyde concentration on the mobility of the I(5, 10) (●— — —●) and I(10, 3) (▲— — —▲) spin probes incorporated into: a and b, intact erythrocytes; c and d, liposomes. (B) The effects of glutaraldehyde concentration on the mobility of the MSL (e) and ISL (f) spin labels incorporated into ghosts.

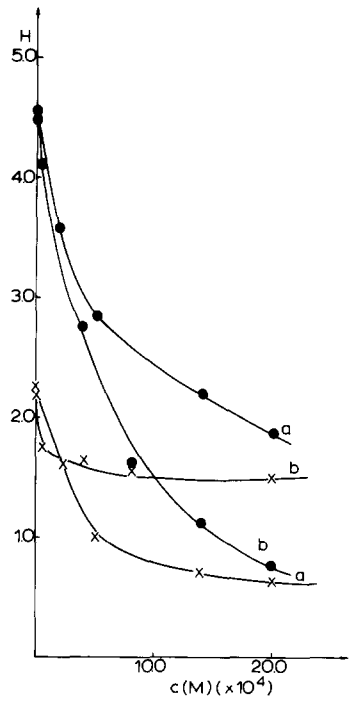


Fig. 4. Immobilization of MSL (×————×) and ISL (●————●) spin labels bound to the ghost membrane with an increased concentration of modifying agents: a, glutaraldehyde and b, OsO_4.

from the influence of both reagents on the liposomes seem to be insignificant.

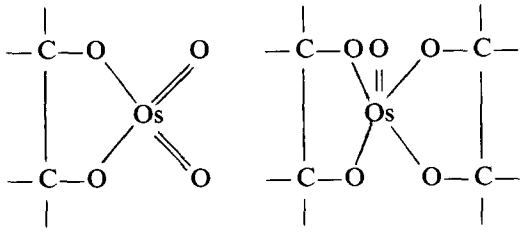
The action of OsO_4 and glutaraldehyde on the intact erythrocytes is much clearer (Figs. 2A and 3A, curves a and b). These agents effectively immobilize the mobility of I(m, n) probes.

Modification of the protein fraction of membrane ghosts has also been observed. The influence of OsO_4 and glutaraldehyde on the mobility of MSL and ISL on proteins is similar. This is illustrated in Figs. 2B and 3B (curves e and f) where the decrease in probe mobility may be seen. However, glutaraldehyde has a more significant effect on the mobility of MSL than on that of ISL. The mobility of MSL and ISL is similarly changed for low concentrations of OsO_4; for higher concentrations the ISL mobility increases more rapidly.

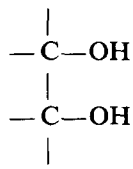
Discussion

According to White et al. [8] the results support a scheme of the reaction of OsO_4 with erythrocyte

membranes in which the double bond of unsaturated lipid is the initial reaction site. It might lead to the formation of Os(VI) derivatives of the type:



In the reductive environment esters are decomposed to form *cis*-diol bonds:



Observed results of the effect of OsO_4 on the mobility of I(m, n) probes in lipid bilayers tend to agree with this suggestion. The osmate esters would be crosslinked to the chains of neighboring lipids. The flexibility of the lipid would decrease and thus so would the mobility of the spin label I(5, 10). The slight increase in I(10, 3) mobility of OsO_4 might be understood if the created *cis*-diols shift to the lipid bilayer polar heads region, as do the alcohols [23].

In Fig. 3A, curve c a weak immobilization of the I(5, 10) probe is observed. This demonstrates that glutaraldehyde might react with lipids and it seems that with $-NH_2$ groups it reacts exclusively. Perhaps Schiff's bases with lipids are formed. This bond between the vicinal $-NH_2$ groups may decrease the flexibility of the lipid bilayer which has been proved to decrease the spin label motion. Nevertheless, the effects of glutaraldehyde and OsO_4 on intact erythrocyte membranes are larger. This occurrence may be explained only by lipid-protein interactions. The first agent, OsO_4 reacts predominantly with the unsaturated lipids [3,8]. The reaction of OsO_4, an oxidizing agent, with intact erythrocyte membrane proteins is improbable [15,16]. On the other hand glutaraldehyde reacts only with the membrane proteins. Since for both agents a large lipid mobility was observed, the changes in the membrane lipid bilayer appear to be caused by deformations of proteins which are interacting with lipids. This modification would change the lipid bilayer as shown in Figs. 2A and 3A (curves a and b).

ISL and MSL probes are bound to the protein fraction of the membranes. ISL reacts with -SH groups of proteins. MSL could bind to -SH as well as to $-NH_2$ [24,26].

Glutaraldehyde modifies the ghost membrane structure, with a more pronounced alteration in the MSL binding site than in the ISL binding site Fig. 3B (curves e and f).

The action of OsO_4 on the ghost membrane is more complicated. The modifications of the lipid fraction would lead to changes in the lipid-protein interactions and herewith the protein conformation can be influenced. On the other hand, OsO_4 might react with -SH groups as an oxidizing agent or with unsaturated protein bonds. The first supposition is realised at low concentrations but the latter only at higher concentrations of OsO_4 (Fig. 2B, curve f).

The presented results can be summarized as follows: both OsO_4 and glutaraldehyde seem to be specific agents, with OsO_4 reacting primarily with lipids and glutaraldehyde primarily with proteins. However, changes in the lipid fraction may induce changes in the state of the protein fraction and vice versa, as can be seen in the EPR spectra.

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