# Analysis of Electron Spin Resonance Spectra of Alkyl Spin Labels in Excised Guinea Pig Dorsal Skin, Its Stratum Corneum, Delipidized Skin and Stratum Corneum Model Lipid Liposomes

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**The electron spin resonance (ESR) spectra of alkyl spin labels were observed in the excised guinea pig dorsal skin, its stratum corneum , delipidized skin and stratum corneum model lipid liposomes. The spectrum of 5-doxylstearic acid (5-NS) in the stratum corneum and order parameter obtained from the spectrum, indicated that the spin label was present in highly ordered lipid lamella. On the other hand, the spectrum of methyl ester of 5-NS (5-NMS) and its apparent rotational correlation time calculated from the spectrum, showed only a weakly immobilized component in the stratum corneum as well as in the whole excised skin. The ester spin label seemed to be scarcely present in the rigid lipid lamella, but mainly in the relatively fluid environment. On the other hand, cationic alkyl spin labels showed quite different spectra depending on their alkyl chain lengths. Long-chain 4-(*N*,*N*-dimethyl-*N*-pentadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl (CAT-15) seemed to be present in the protein region of the stratum corneum as we recently reported, whereas hydrophilic quaternary ammonium spin label 4-trimethylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl (CAT-1) seemed to be present in the bulk water of the skin, even in delipidized skin. These findings indicated that the different interaction and different localization of the alkyl spin labels depended on their electronic charge as well as their alkyl chain lengths.**

**Key words** spin label; ESR; skin; stratum corneum; liposome

Electron spin resonance (ESR) analysis by spin labels has been developed as a valuable method in the study of physico-chemical properties of various biological membranes.[1,2] It has also been applied to the skin study to examine the physico-chemical properties of stratum corneum and their changes by addition of different kinds of absorption enhancers.[3—5] Although a lot of spin labels have been used for these purposes,[3—6] localization of the spin labels in the skin as well as in the biological membranes is unclear. The ESR spectrum of each spin label gives information about the motional character of the spin label in the particular environment in which it is mainly present. Fatty acid spin labels such as 5-doxylstearic acid (5-NS) have been reported to be present in the lipid lamella in human stratum corneum.[7,8] On the other hand, our recent analysis on the ESR spectra of a long-chain quaternary ammonium spin label 4-(*N*,*N*-dimethyl-*N*-pentadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl (CAT-15) in excised guinea pig dorsal skin, has suggested that the spin label is mainly present by binding to stratum corneum proteins.[6] These results suggested the possibility that the localization of the spin labels may be different depending on their electric charge and their chemical structure.

To obtain the information on the localization of different kinds of spin labels in the skin, and their interaction with the skin components, in this study we observed ESR spectra of anionic 5-NS, nonionic its methyl ester, 5-doxylmethylstearate (5-NMS), cationic CAT-15 and a short-chain analog of CAT-15 4-trimethylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl (CAT-1), whose structures are shown in Fig. 1. We observed ESR spectra of these spin labels in three different skin preparations: excised guinea pig dorsal skin, its stratum corneum and delipidized skin. To compare them with those in the skin preparations mentioned above, we further observed the ESR spectra of the same spin labels in stratum corneum model lipid liposomes (prepared by a sonication with a probe-type sonicator), whose physico-chemical properties we reported previously.[9] From these observations, we tried to clarify the dependence of the localization of the spin labels in the skin and that of their interaction with skin components on their electric charge, as well as on their alkyl chain lengths. We tried to reveal the importance of the selection of the spin labels to obtain the information on the physico-chemical properties of specific regions of the skin.

## Experimental

**Materials** 5-NS was purchased from Aldrich (Milwaukee, WI, U.S.A.). 5-NMS, ceramide (type IV), cholesterol and cholesteryl sulfate were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Iodide salts of CAT-1 and CAT-15 were from Molecular Probes, Inc. (Junction City, OR, U.S.A.). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

**Skin Preparations** Full thickness dorsal skin was excised from male guinea pigs and subcutaneous fat and other extraneous tissues were trimmed. The stratum corneum sheets were separated by trypsination from the excised skin as described previously.[6] The skin was placed stratum corneum side up, and floated on 0.2% trypsin in phosphate buffered saline (PBS, pH 7.4) at 37 °C for 12 h. Stratum corneum lipids were extracted by incubating the excised skin with a chloroform–methanol mixture (2 : 1 vol)

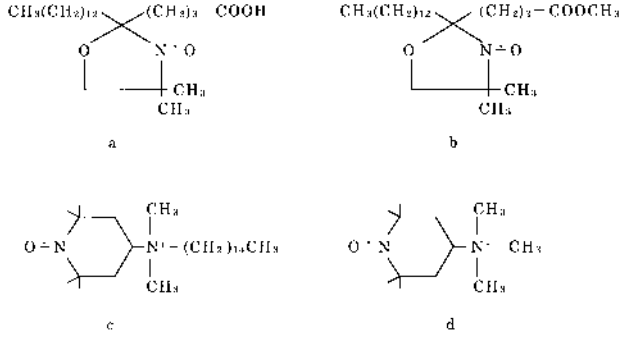


Fig. 1. Structures of Spin Labels Used in This Study; 5-NS (a), 5-NMS (b), CAT-15 (c) and CAT-1 (d)

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for 12 h and washing it extensively with PBS as described previously.[10]

**Preparation of Stratum Corneum Model Lipid Liposomes** Ceramide, cholesterol, cholesterol sulfate and palmitic acid were dissolved in chloroform at a weight ratio of 4.0 : 2.5 : 1.0 : 2.5 (molar ratio, 3.2 : 3.2 : 1.0 : 4.4), as described previously.[9] The solvent was evaporated, the lipids were suspended in PBS and multilamellar vesicles were prepared by vortex mixing. The total lipid concentration was 1.2 mM. Then, the vesicle suspension was sonicated with a probe-type sonicator at 70 °C for 10 min at an output power of 80W under a stream of nitrogen.

**Measurement of ESR Spectra** Measurement of ESR spectra was carried out for spin-labeled slices of skin preparations. Both the epidermis side and the dermis side of the skin were incubated with PBS at 37°C. After removing PBS from the epidermis side (area 0.65 $cm^2$), 200 $\mu$l of 50 $\mu$M spin labels in PBS was added and incubated for 2 h at 37 °C. Incubation of the stratum corneum and delipidized skin with the spin label was carried out similarly. The spin label solution left on the epidermis or the stratum corneum was removed by washing with PBS. Slices of the spin-labeled skin samples were inserted into quartz ESR tubes (Labotech, Tokyo).

Measurement of ESR spectra for the stratum corneum model lipid liposomes was carried out by using spin-labeled liposome suspension. The stratum corneum lipid liposome suspension was incubated with 25 $\mu$M spin labels at 37 °C for 2 min and transferred to duplicate 20 $\mu$l capillaries. One end of the capillaries was sealed with Hematoseal (Terumo, Tokyo, Japan) and inserted into the ESR tubes.

ESR spectra were measured at 32 °C with a TE-200 (X-band) spectrometer (JEOL, Tokyo, Japan) with 100 kHz field modulation frequency at an out-put power of 8 mW as reported previously.[5] 0.1 mT modulation amplitude was used for CAT-1 in all preparations, 5-NMS in excised skin and stratum corneum, and CAT-15 in liposomes. 0.2 mT modulation amplitude was used for other samples. Order parameter and apparent rotational correlation time were obtained from the spectra as described.[11]

## Results

**ESR Spectra of 5-NS** The ESR spectrum of 5-NS in the stratum corneum (Fig. 2b) showed a similar spectrum with that in the stratum corneum model lipid liposomes (Fig. 2d). The order parameter in Table 1 obtained from the ESR spectra gave a typical value, which indicated the presence of the spin label in highly ordered lipid lamella in the stratum corneum. This is consistent with the previous findings in human stratum corneum.[7,8] Order parameter of 5-NS in the stratum corneum was slightly larger than that in the stratum corneum model lipid liposomes (Table 1). As previously reported,[5] the spectrum of the same spin label in the original excised skin (Fig. 2a) indicated the presence of a weakly immobilized component, in addition to the strongly immobilized component; this seemed to correspond to the spin label in the rigid lipid lamella as was also revealed in the parameter values shown in Table 1. On the other hand, ESR spectrum of 5-NS in delipidized skin showed that the spin label was present in a solid circumstance (Fig. 2c).

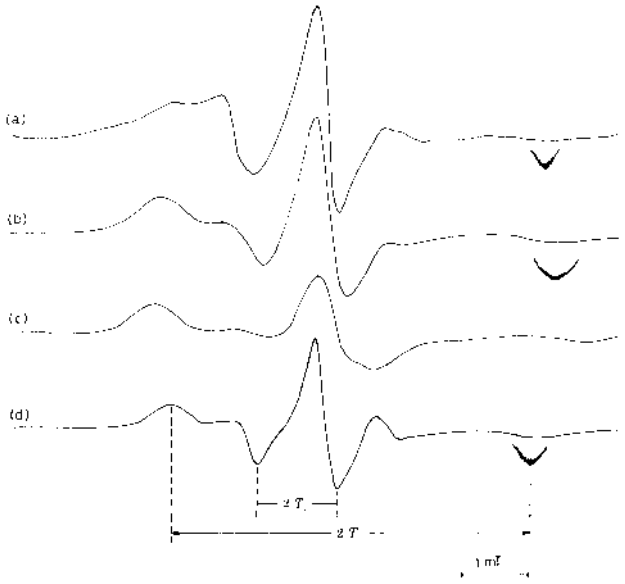


Fig. 2. ESR Spectra of 5-NS in Excised Guinea Pig Dorsal Skin (a), Its Stratum Corneum (b), Delipidized Skin (c) and Stratum Corneum Lipid Liposomes (d)

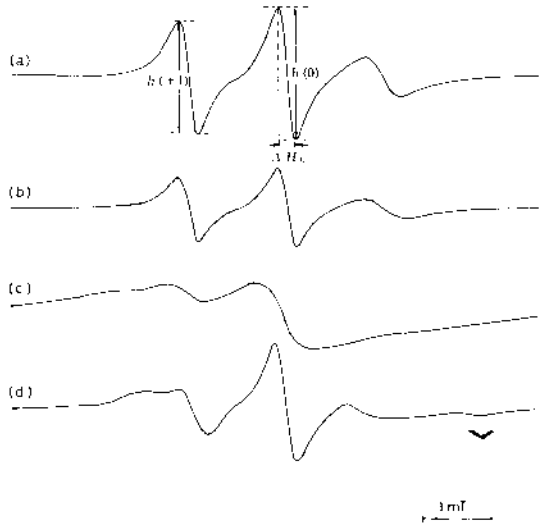


Fig. 3. ESR Spectra of 5-NMS in Excised Guinea Pig Dorsal Skin (a), Its Stratum Corneum (b), Delipidized Skin (c) and Stratum Corneum Lipid Liposomes (d)

**ESR Spectra of Methyl Ester of 5-NS** On the other hand, the spectra of methyl ester of 5-NS (5-NMS), and its apparent rotational correlation times obtained from the spectra, which are shown in Fig. 3 and Table 1, respectively, had a weakly immobilized component alone in both original excised skin and its stratum corneum. In contrast, the spin label showed a different spectrum in the stratum corneum model

Table 1. Order Parameters ($S$) and Apparent Rotational Correlation Times ($\tau_o$) of 5-NS, Its Methyl Ester (5-NMS) and CAT-15 in Excised Guinea Pig Dorsal Skin, Its Stratum Corneum and Stratum Corneum Model Lipid Liposomes at 32 °C

| Sample | 5-NS | | 5-NMS | | CAT-15 |
|---|---|---|---|---|---|
| | $S^{a)}$ | $\tau_o$ ($\times 10^{-9}$ s)$^{a)}$ | $S^{a)}$ | $\tau_o$ ($\times 10^{-9}$ s)$^{a)}$ | $\tau_o$ ($\times 10^{-9}$ s)$^{a)}$ |
| Excised skin | 0.656±0.009 | 1.82±0.14 | —$^{b)}$ | 1.85±0.05 | —$^{b)}$ |
| Stratum corneum | 0.698±0.004 | —$^{b)}$ | —$^{b)}$ | 1.91±0.04 | —$^{b)}$ |
| Liposomes | 0.666±0.004 | —$^{b)}$ | 0.540±0.008 | 3.30±0.16 | 2.54±0.08 |

*a*) Data are means±S.D. of four experiments. Order parameters were calculated by measuring the outer and inner hyperfine splittings, 2 $T_{\parallel}$ and 2 $T_{\perp}$, from the ESR spectra as shown in Fig. 2d. Apparent rotational correlation times were calculated by measuring the central peak width, $\Delta H_0$, central peak height, $h$ (0), and peak height of lower magnetic fields, $h$ (+1), from the ESR spectra as shown in Fig. 3a. *b*) Not measured from the spectra.

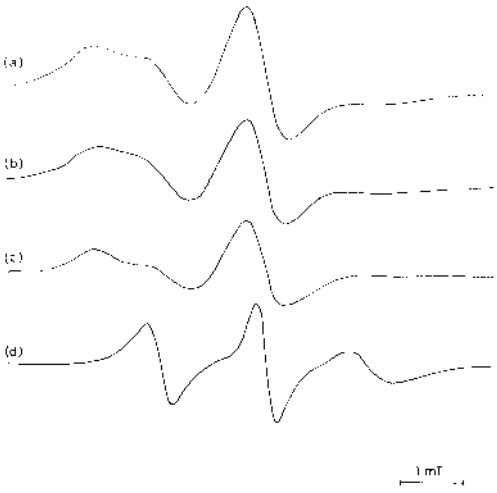


Fig. 4. ESR Spectra of CAT-15 in Excised Guinea Pig Dorsal Skin (a), Its Stratum Corneum (b), Delipidized Skin (c) and Stratum Corneum Lipid Liposomes (d)

Spectra of CAT-15 in excised skin and stratum corneum were reported in the previous paper[6] and shown here again.

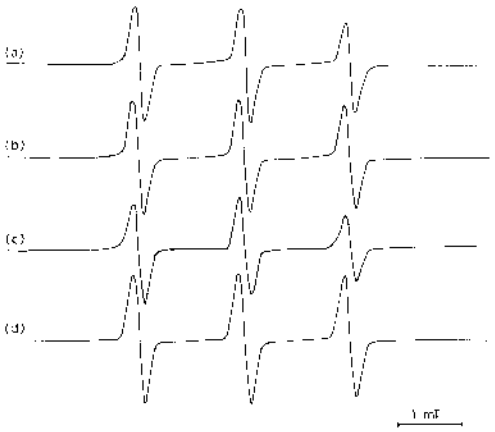


Fig. 5. ESR Spectra of CAT-1 in Excised Guinea Pig Dorsal Skin (a), Its Stratum Corneum (b), Delipidized Skin (c) and Stratum Corneum Lipid Liposomes (d)

lipid liposomes (Fig. 3d), which indicated the presence of the spin label in a highly ordered environment. The broad signal of this ester spin label in delipidized skin, shown in Fig. 3c, differed in its shape from those in the original excised skin and its stratum corneum. It was also different from that in the stratum corneum lipid liposomes. Similar results were observed for other fatty acid ester spin labels (data not shown).

**ESR Spectra of Quaternary Ammonium Spin Labels** We have reported that long-chain quaternary alkylammonium spin label CAT-15 is present in a solid state in both excised skin and its stratum corneum, according to the analysis of ESR spectra,[6] which were shown again in Figs. 4a and 4b. ESR spectra of CAT-15 in delipidized skin (Fig. 4c) resembled those in the original skin and its stratum corneum. Molecular motion of the spin label in the skin preparations (Figs. 4a, 4b, 4c) were much more restricted compared with that in the stratum corneum lipid liposomes (Fig. 4d), which indicated that the spin label was not present in lipid lamella but in a nearly solid circumstance in these skin preparations.

In this work we also observed the ESR spectra of hydrophilic short-chain CAT-1 to see the effect of the alkyl chain length. As shown in Fig. 5, isotropic signals were observed in the excised skin, its stratum corneum and delipidized skin as well as in stratum corneum lipid liposomes. These findings greatly differed from those of the long-chain CAT-15.

## Discussion

ESR analysis has been applied to the studies of biological membranes, including skin. However, there have been few studies on the dependence of the interaction of the spin labels with the membrane components and their localization in the membrane on their chemical structure and electrical charge. As previously reported in human stratum corneum,[7,8] 5-NS in the stratum corneum of guinea pig dorsal skin showed a typical spectrum, which indicated the presence of the spin label in gel state lipid lamella. Intercellular lipid lamella in stratum corneum is known to have a highly ordered structure and to work as an effective barrier to penetration of chemical substances.[12] It has also been clarified that free fatty acids, such as palmitic acid, are present as one of the major components in the stratum corneum lipid lamella.[13] Therefore, the fatty acid spin label seems to be inserted into the lipid lamella like those free fatty acids. The strongly immobilized component of the same spin label in the original excised skin seems to correspond to the spin label in the rigid lipid lamella mentioned above. On the other hand, the weakly immobilized component in the excised skin seemed to be due to localized 5-NS in a fluid bilayer-like environment; this may be associated with the spin labels that are in a polar environment on the skin surface[14] or in an interface region between bulk water and lipid lamella.

The smaller value of the order parameter of the strongly immobilized component of 5-NS in the original skin may be due to the incomplete resolution of the two components in the spectrum. Although the order parameter of 5-NS in the model lipid liposomes was also smaller than that in the stratum corneum, it may be due to the contribution of other lipid components in the stratum corneum lipid lamella such as ceramide 1. Ceramide 1 is absent in the model lipid liposomes and has been known to have an important role in making a highly ordered structure of the lipid lamella in the stratum corneum of the skin.[15]

Different from the fatty acid spin label, the methyl ester spin label 5-NMS had only an immobilized component in both original excised skin and its stratum corneum. The ester spin label seems to be mostly present in a relatively fluid environment of the stratum corneum and scarcely present in the rigid lipid lamella, although the detailed localized region of the ester spin label in the skin as well as the reason of the preference for the fluid environment is still unclear. The broad signal in the ESR spectrum of 5-NMS in delipidized skin suggested that this spin label was also present in a solid circumstance there, just like 5-NS. In delipidized skin, these spin labels seem to be bound to the skin proteins. Since these spin labels seem to significantly bind to the skin proteins only in delipidized skin, their affinity to the lipid regions in the skin are probably much larger than that to the skin proteins.

ESR spectra of long-chain quaternary alkylammonium spin labels in biological membranes, such as erythrocyte

membrane, have been revealed to arise from their motions in protein regions as well as those in lipid regions.[16] In skin, long-chain quaternary alkylammonium spin label, CAT-15, seems to be present by binding to proteins in the stratum corneum, probably keratin fiber, as revealed previously.[6] In contrast, hydrophilic quaternary ammonium spin label, CAT-1, showed the isotropic motion and seems to be present in the bulk water in the skin. Therefore, the presence of the long alkyl chain is essential for the interaction with the skin proteins. Unlike the fatty acid spin labels and their ester spin labels, the affinity of the long-chain cationic spin label to the skin proteins are probably much larger than that to the lipids.

The present findings indicated that the different interaction and different localization of alkyl spin labels in skin depend on their electronic charge and alkyl chain lengths. Therefore, the selection of the spin labels is necessary to reveal the physico-chemical properties of the specific region of the skin. Furthermore, the combinational use of these alkyl spin labels is expected to give useful information on the properties of different regions of the skin and their changes by the addition of various drugs, cosmetics and absorption enhancers. The use of other spin labels, such as fatty acid spin labels with different positions of labeling and cholesterol spin labels, may also give additional information on these matters.

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