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Disk to Sphere Transformation of Erythrocyte induced by 1-Anilino-8-Naphthalene Sulfonate (ANS). II.[1] Quantitative Study on Adsorption of ANS by Whole Erythrocyte and by Ghost Membrane

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Amounts of 1-anilino-8-naphthalene sulfonate (ANS) adsorbed by whole bovine erythrocyte and by its ghost membrane were studied at ANS concentration from 0.01 to 3 mM. For whole erythrocyte the amount is nearly proportional to the free ANS concentration with the ratio of 1.1×10 [mole/*l*-packed-erythrocyte]/[mole/*l*-medium]. The ghost membrane has two kinds of binding site for ANS.

The dissociation constants and the numbers of binding sites are calculated as follows: $K_I = 9.1 \times 10^{-5}$ M and $n_I = 1.9 \times 10^{-2}$ mole/kg-membrane for site I; $K_{II} = 1.0 \times 10^{-2}$ M and $n_{II} = 5.8 \times 10^{-1}$ mole/kg-membrane for site II. The binding site I and II can be attributed to protein and lipid, respectively, judging from membrane mass per mole of sites. The mechanism of disk to sphere transformation was discussed from these results.

Norml mammalian erythrocytes are known to cause morphological changes by the addition of some agents,[3] by decreasing the adenosine triphosphate (ATP) concentration in erythrocyte,[4] by the change of pH in surrounding media,[5] or by increasing lysolecithin concentration in the plasma.[6]

It has been shown in a previous work that the agent 1-anilino-8-naphthalene sulfonate (ANS) induces a transformation of bovine erythrocyte from a biconcave disk into a smooth sphere through a crenated sphere.[1] This transformation may be attributed to a change in the structure of cellular membrane. On the other hand, it was reported that the binding of this agent affects ionic permeability of the membrane in the same range of ANS concentration.[7] In order to clarify the mechanism of these phenomena it is necessary to obtain the knowledge on the amount of ANS adsorbed by the membrane. Although several mesurements were performed on the amount of ANS adsorbed by erythrocyte membrane, the ranges of free ANS concentration studied are lower than the concentration where the transformation occurs.[8]

In the present work the amount of ANS adsorbed by intact erythrocyte and ghost membrane were measured in the range of free ANS concentration from 0.01 to 3 mM. The studies on ghost were performed to measure the adsorption of ANS to membrane more precisely. The mechanism of the transformation was discussed from the properties of the binding sites.

Experimental

Erythrocytes were centrifuged from fresh bovine blood anticoagulated with acid-citrate-dextrose. The precipitate was resuspended in the medium, 150 mM NaCl, 5 mM phosphate buffer, pH 7.0, and washed twice.

1) Part I: S. Yoshida and A. Ikegami, *Biochem. Biophys. Acta*, 367, 39 (1974).
2) Location: *Hongo, Bunkyo-ku, Tokyo, 113, Japan.*
3) B. Deuticke, *Biochim. Biophys. Acta*, 163, 494 (1968).
4) M. Nakao, T. Nakao, S. Yamazone, and K. Yoshikawa, *J. Biochem.* (*Tokyo*), 49, 487 (1961).
5) E. Ponder, "Hemolysis and Related Phenomena," Grune and Stratton, New York, 1946, pp. 10—49.
6) T. Sato, *Chem. Pharm. Bull.* (Tokyo), 21, 176 (1973).
7) P.A.G. Fortes, Ph.D. Thesis, Univ. Pennsylvania, 1972.
8) B. Rubalcava, D.M. de Munoz, and G. Gilter, *Biochem.*, 8, 2742 (1969); R.B. Freedman and G.K. Radda, *FEBS LETTERS*, 3, 150 (1969); M.B. Feinstein, L. Spero, and H. Felsenfield, *FEBS LETTERS*, 6, 245 (1970).

The final concentration of erythrocyte was about 30 per cent in volume. Ghost was prepared according to the method of Dodge, *et al.*,[9] washed three times and suspended in the medium. The final concentration of ghost was 10 mg per ml. Erythrocyte and ghost were treated at 4° throughout the study.

The amount of ANS adsorbed by whole erythrocyte was determined by the measurements of free ANS concentration in the medium. An aliquot of 1 mM or 10 mM ANS solution adjusted to be isotonic by the addition of NaCl was mixed with 3 or 4 ml of erythrocyte suspension. At time intervals of incubation at 4° the suspension was centrifuged at 1×10^4 *g* for 20 minutes to spin down not only intact erythrocyte but also membrane fragments produced by partial hemolysis. The concentration of ANS in the supernatant was determined from the optical density at 350 nm, where the correction was made for the absorbance attributed to a trace of hemoglobin in the suppernatant.

The amount of ANS adsorbed by ghost membrane was determined by the same way as mentioned above with a slight modification; centrifugation was made at 7×10^4 *g* for 30 minutes.

Results

Adsorption of ANS by Whole Erythrocyte

Time course of adsorption of ANS by whole erythrocyte was measured at initial free ANS concentration of 7.4×10^{-4}M. ANS concentrations in the supernatant are plotted against time in Fig. 1. Equilibrium is reached after 8 hours of incubation. The solid curve was

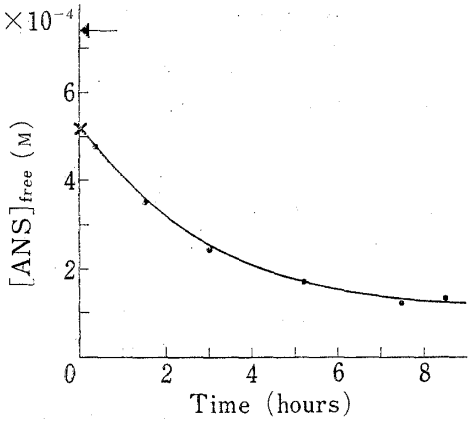

Fig. 1. Time Course of ANS Uptake by Erythrocyte

Initial ANS concentration is 7.4×10^{-4}M (←). The solid curve was derived assuming that the ANS concentration reaches the equiribrium value exponentially with the relaxation time of 3.0 hours. ×: the extraporated value to time of zero.

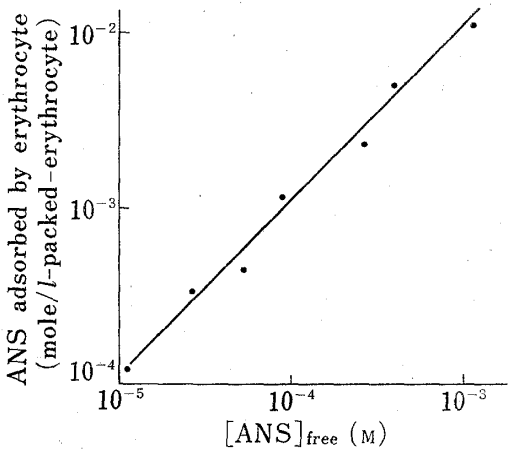

Fig. 2. The Amount of ANS adsorbed by Intact Erythrocyte

It is proportional to the free ANS concentration with the ratio of 1.1×10 [mole/*l*-packed-erythrocyte]/[mole/*l*-medium].

calculated assuming that the ANS concentration reaches the equilibrium exponentially with the relaxation time of 3.0 hours. Experimental values agree well with the calculated. The mark "X" in Fig. 1 represents the extraporated value to time of zero. The disagreement between the initial concentration (7.4×10^{-4}M) and the extraporated value (5.2×10^{-4}M) indicates that there is another rapid process of adsorption of ANS.

The amount of ANS adosorbed by whole erythrocyte in equilibrium was measured after 8 hours of incubation for various free ANS concentrations. Results are shown in Fig. 2, where the amount of adorbed ANS is expressed in the unit of mole per liter of packed erythrocyte. The amount of adsorbed ANS is proportional to free ANS in the suspending medium with a proportional coefficient of 1.1×10 [mole/*l*-packed-erythrocyte]/[mole/*l*-medium].

Adsorption of ANS by Ghost Membrane

The amount of ANS adsorbed by ghost membrane in equilibrium was measured after 1 hour of incubation over the range of free ANS concentration from 0.01 to 3 mM. It was

9) J.T. Dodge, C. Mitchell, and D.J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).

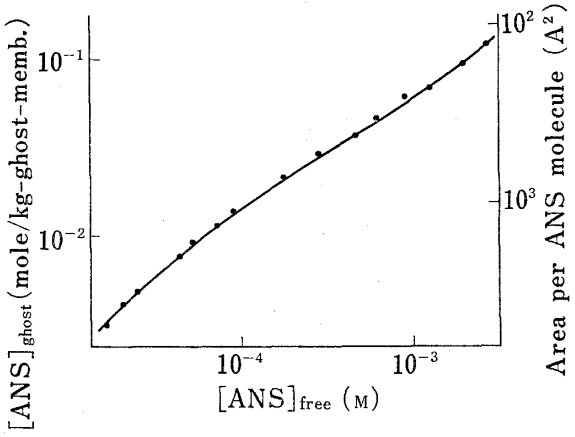

Fig. 3. The Amount of ANS adsorbed by Ghost Membrane

The amount of adsorbed ANS is expressed as moles of bound ANS per kg of dry ghost membrane on the left scale. The scale on the right side is marked with membrane area associated with an ANS molecule.

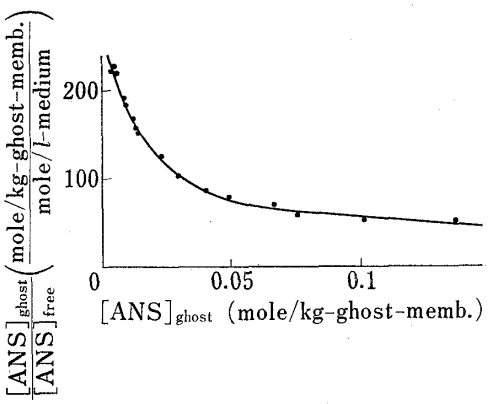

Fig. 4. The Scatchard Plot of the Amount of ANS adsorbed by Ghost Membrane

Assuming two kinds of binding site, I and II, the dissociation constants and the numbers of binding site were determined with a least squares method as follows: $K_I=9.1\times10^{-6}$M, $n_I=1.9\times10^{-2}$ mole/kg-ghost-membrane, $K_{II}=1.0\times10^{-2}$M, and $n_{II}=5.8\times10^{-1}$ mole/kg-ghost-membrane. The solid curve was calculated with these parameters.

ascertained beforehand that after fifteen minutes to two hours of incubation there was not any difference in the amount of adsorption, that is, no relaxation process was observed.

The results are shown in Fig. 3, where the amounts adsorbed by the ghost membrane are plotted against the free ANS concentration. In the analysis it is assumed that the free ANS concentration inside of ghost is the same with that in the outside medium (see Discussion). The scale on the right side in Fig. 3 is expressed as membrane area associated with an adsorbed ANS molecule. In this conversion the ratio between dry weight[9] and membrane area[10] (surface density) of a human erythrocyte, 1.0×10^{-6} g/cm^2, is used on the assumption that the surface density of membrane of a human erythrocyte is the same with that of a bovine erythrocyte. The average distance between the nearest neighbouring ANS molecules adsorbed by the membrane is larger than 10A in the concentration range of ANS examined. As Debye-Hückel radius of the suspending medium is about 6.7A, electrostatic repulsion between ANS molecules must be partially screened by ionic atmosphere. Neglecting the electrostatic effect, the results were analysed with Scatchard plot[11] as shown in Fig. 4. For one kind of binding site the plot should be expressed by a straight line as following equation:

$$\frac{[ANS]_{ghost}}{[ANS]_{free}}=\frac{1}{K}(n-[ANS]_{ghost}) \tag{1}$$

K is the dissociation constant, n the number of binding sites per unit mass of ghost membrane, and $[ANS]_{ghost}$ the number of ANS molecules adsorbed by unit mass of ghost membrane. The results in Fig. 4 cannot be fitted into a straight line. This indicates the existence of more than two kinds of binding site. Assuming two kinds of binding site (I or II) the equation (1) should be replaced by following ones (i=I or II):

$$[ANS]_{ghost}=[ANS]_{ghost,\,I}+[ANS]_{ghost,\,II} \tag{2}$$

and

$$\frac{[ANS]_{ghost,\,i}}{[ANS]_{free}}=\frac{1}{K_i}(n_i-[ANS]_{ghost,\,i}) \tag{3}$$

10) P.B. Canhan, *J. Theor. Biol.*, **26**, 61 (1970).
11) P. Seeman, S. Roth, and H. Schneider, *Bioch. Biophys. Acta*, **225**, 171 (1971).

The dissociation constants, K_I and K_{II}, and the numbers of binding site, n_I and n_{II}, were determined by a least squares method as follows: $K_I=9.1\times10^{-5}$M, $n_I=1.9\times10^{-2}$ mole/kg-ghost-membrane, $K_{II}=1.0\times10^{-2}$M, and $n_{II}=5.8\times10^{-1}$ mole/kg-ghost-membrane. The solid curve in Fig. 4 was calculated with these parameters and it shows close agreement with experimental values. The parameters K_I and n_I are compatible for those of human or bovine erythrocyte determined by fluorometric titration at lower ANS concentration.

Discussion

In an effort to elucidate the mechanism of disk to sphere transformation of erythrocyte induced by ANS was made a quantitative study. As a model of erythrocyte it is assumed that whole erythrocyte is composed of membrane and interior matter and that the properties of the membrane of intact erythrocyte are the same with those of the ghost membrane.

The amount of ANS adsorbed by whole erythrocyte and by ghost membrane (Fig. 2 and 3) are converted to the amount associated with one erythrocyte and the results are shown in Fig. 5. In the calculation the volume and the surface area of a bovine erythrocyte is taken to be 58 μ^3 and 95 μ^2, respectively.[12] The ratio between the amount of ANS adsorbed by ghost membrane and by whole erythrocyte is 1/3 and 1/12 at the lowest and at the highest concentration, respectively.

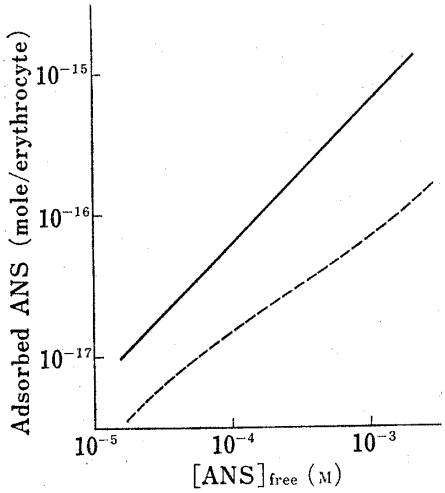

Fig. 5. The Amount of ANS adsorbed by Whole Erythrocyte and by Ghost Membrane per Erythrocyte

——: whole erythrocyte
------: ghost membrane

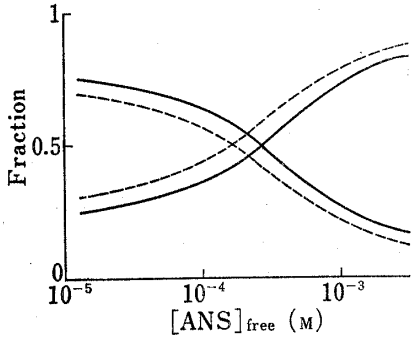

Fig. 6. The Fraction of ANS bound to Site I and II

——: excluding the electrostatic effect
------: including the electrostatic effect

While it takes about 8 hours for the amount of adsorbed ANS to reach the equiribrium for intact erythrocyte, it does less than 15 minutes for ghost membrane. The results shown in Fig. 1 indicate further that there are at least two processes for the adsorption by erythrocyte. The amount of ANS adsorbed in the rapid process is calculated to be 2.4×10^{-17} mole/erythrocyte. On the other hand, ANS adsorbed by ghost membrane at the extrapolated free ANS concentration (5.1×10^{-4}M as shown in Fig. 1) is calculated to be 4.2×10^{-17} mole/ghost from Fig. 5. There is fair agreement between both the values.

These results can be explained consistently as follows: When ANS is added to the erythrocyte suspension, one part of it is adsorbed by the membrane and another penetrates

12) W. Gruber and B. Deuticke, *J. Membrane Biol.*, **13**, 19 (1973).

the membrane into the interior of the erythrocyte and is adsorbed by the interior matter which is mainly hemoglobin. The former corresponds to the rapid process and the latter is controlled by the membrane permeation of ANS molecules and corresponds to the slow pocess. As the membrane of intact erythrocyte is permeable to ANS molecules, the ghost membrane which are considered to be more fragile may be also permeable. But the relaxation porcess for ANS molecules to permeate the membrane into the inside of ghost was not obserbed. This is because the reseal of ghost in the preparation is imperfect and the membrane is porous. So the ANS concentration of the inside becomes the same instantly with that of the outside.

The disk to sphere transformation induced by ANS accomplishes whithin 30 seconds. This means that the transformation may be attributed to the rapid process, that is, to the erythrocyte membrane.

As ghost membrane has two kinds of binding site, whole erythrocyte should have more than three kinds, that is, two kinds on the membrane and one kind in the interior. The accuracy of the experiment for whole erythrocyte was, however, not compatible with the analysis to decompose binding sites into multiple kinds of site.

The ghost membrane has two kinds of binding site of ANS. The membrane mass associated with a mole of sites is calculated to be 5.3×10^4 g and 1.7×10^3 g for site I and II, respectively, from the number of binding sites, n_{I} and n_{II}. The membrane mass associated with one site of I is roughly corresponds to the molecular weight of subunits of usual proteins observed in erythrocyte membrane and is lower than molecular weight of lipids. So the site I may be attributed to proteins. Similarly the site II may be attributed to lipid, though the possibility cannot be eliminated that the site II is composed of proteins with multiple binding sites on one molecule. This conclusion agrees with the results obtained by nanosecond fluorescence studies on ghost membrane by Fortes:[7] ANS molecules are estimated to be bound to two kinds of site of different environment, protein and lipid.

The fraction of adsorbed molecule by each kind of site was calculated from the above parameters and the results are shown by solid curves in Fig. 6. It is shown that site I is dominant at lower concentration of ANS where crenations elongate, and that site II is dominant at higher concentration of ANS where crenations flatten out. Therefore site I and II may be attributed to elongation and flattening out of crenations, respectively.

Recently Lesslaufer, *et al.*[13] reported the X-ray diffraction studies on a lecithin/cardiolipin bimolecular model membrane with incorporated ANS. When the composition in the membrane is 1 ANS molecule per 3 fatty acid residues, the thickness of the membrane decreases from 54.0 to 39.0 Å, that is, the configuration change of lipid membrane occurs. At higher free ANS concentration the composition of ANS which occupies site II becomes the same order with the value mentioned above. Therefore if the binding site is composed of lipid, it is probable that the transformation of the erythrocyte membrane occurs at higher ANS concentration.

The mechanism of the transformation is supposed as follows: Erythrocyte membrane is in a mosaic state of lipid and protein which has been proposed as a mosaic model.[14] When ANS is added to erythrocyte suspension, some of them are bound to protein in the membrane. Then the conformation of protein or the interaction between lipid and protein is affected and the expansion of the surface area of erythrocyte membrane may occur. The crenations grow under the restriction of the constant volume. When ANS molecules are added further, the other kind of sites which may be lipid is occupied by ANS molecules. Then the structure of lipid bilayer is disturbed and proteins are repelled out to the surface of the membrane. As a result contraction of membrane area occurs and crenations flatten out. The shape becomes spherical at the final stage.

13) W. Lesslaufer, J. Cain, and J.K. Blasie, *Biochim. Biophys. Acta*, **241**, 547 (1971).
14) S.J. Singer and G.L. Nicolson, *Science*, **175**, 720 (1972).

Appendix

Taking into account the effect of electrostatic repulsion between ANS molecules, the results are analysed all over again. The free ANS concentration at the surface of the membrane, $[ANS]_{sf}$, can be expressed as follows:

$$[ANS]_{sf} = [ANS]_{free} \exp(e\psi/kT) \tag{4}$$

where ψ is electrostatic potential. According to the theory of electro-chemical double layer,[15] ψ is given by the expression:

$$\sinh\left(\frac{e\psi}{2kT}\right) = (\sigma_0+\sigma_{ANS})\left(\frac{\pi}{2N\varepsilon kT}\right)^{1/2} \tag{5}$$

where σ_0 is the surface charge density normally present on the membrane and σ_{ANS} is that of the bound ANS ions, N is number density of the ions, mostly Na^+ and Cl^-, far from the membrane, and ε is a dielectric constant of the medium. σ_{ANS} is given by the expression:

$$\sigma_{ANS} = -[ANS]_{ghost}/d \tag{6}$$

where d is the surface density of ghost membrane. Note that the charge density, σ_{ANS}, has a negative value because of negative charge of ANS ions. It is assumed further that the potential is not so very large that the left hand side of eq. (4) can be replaced by $e\psi/2kT$. The Scatchard plot is then modified as shown by the equations:

$$\frac{[ANS]_{ghost}}{[ANS]_{free}} \exp(e\psi/kT) = \frac{1}{K}(n-[ANS]_{ghost}) \tag{7}$$

and

$$e\psi/kT = (\sigma_0+\sigma_{ANS})\left(\frac{2\pi}{N\varepsilon kT}\right)^{1/2} \tag{8}$$

With these equations the parameters of binding sites were calculated over again: For site I, the dissociation constant $K_I' = 7.3\times10^{-5}$ M, and the number of binding site $n_I' = 1.5\times10^{-2}$ mole/kg-ghost-membrane; for site II, the number of binding site is infinitive and the partition coefficient $P_{II}' = 7.5\times10$ [mole/kg-ghost-membrane]/[mole/l-medium]. In the above calculation the charge density of the bovine erythrocyte membrane, σ_0, is estimated to be $-6.4\times10^{12}/cm^2$ from experiments of electrophoresis.[16] The value of surface density, d, is 1.0×10^{-6} g/cm^2 as described in Results.

The dashed curves in Fig. 6 were obtained by the analysis. No essential difference exists between the two analysis and no great modification may be necessary in Discussion.

Acknowledgement Thanks are given to Professor A. Ikegami and Dr. A. Sakanishi for helpful discussion and to Dr. K. Kinoshita for computer calculation.

15) J. Yguerabide, "Fluorescence Techniques in Cell Biology," ed. by A.A. Thaer and M. Sernetz, Springer-Verlag, Berlin-Heidelberg-New York, 1973, pp. 325—331.
16) E.H. Eylar, M.A. Madoff, O.V. Brody, and J.L. Oncley, *J. Biol. Chem.*, 237, 1992 (1962).