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Anesthetic-Protein Interaction: Surface Potential of Bovine Serum Albumin Estimated by a pH-Sensitive Dye

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SUMMARY

It is often contended that inhalation anesthetics act on proteins via perturbation of lipid membranes. However, direct interaction between anesthetics and water-soluble proteins also has been demonstrated. We postulate that the anesthetic action is directed to the interface between water and macromolecules, irrespective of lipid membranes or proteins. The present study deals with anesthetic effects upon interfacial properties of a water-soluble, crystalline delipidated bovine serum albumin. A pH-indicator dye, bromothymol blue, was used to probe the surface potential of the protein. When a pH-indicator dye binds to a macromolecule, the pH, indicated by the color of the dye, differs from the bulk pH measured by a pH meter. This is because 1) the pH of the microscopic area, where the dye is adsorbed, differs from the bulk due to the surface electrostatic potential that interacts with hydrogen ions (electrostatic terms), and 2) the physical property that affects the color of the dye at the bound region is different from the bulk (nonelectrostatic terms). The mismatch between the bulk pH and the color of the bound pH indicator can be used to probe the property of the dye binding site. By screening the electrostatic effects with high ionic strength, the anesthetic effects upon the nonelectrostatic term were shown to be negligible under the present experimental conditions; the pH-color mismatch was mainly caused by the anesthetic effect upon the electrostatic potential of the macromolecular surface interacting with the dye. Accordingly, the surface potential of the dye binding site was estimated from the mismatch. It was found that inhalation anesthetics decreased the surface potential. The partial pressures of diethylether, enflurane, and methoxyflurane that decreased the surface potential by 10 mV were $2.1 \cdot 10^{-2}$, $1.7 \cdot 10^{-2}$, and $0.17 \cdot 10^{-2}$ bar, respectively, which were in agreement with the minimal alveolar concentrations of these anesthetics to achieve surgical anesthesia.

Anesthesia research is unique in the sense that, although most drugs are either agonists or antagonists of the specific receptors, their anesthetic actions appear to be nonspecific. When Cullen and Gross (1) successfully anesthetized a man by xenon at atmospheric pressure, the nonspecificity of anesthetic action appeared to be established. Butler (2) stated: "If the inert gases can be considered as true anesthetics, the action of these spherically symmetrical atoms without any permanent dipoles furnished the most conclusive demonstration that anesthesia need not depend on the effects of any specific structural groupings." Featherstone and Muelbaecher (3) also contended that the action of anesthetics does not involve any specific receptor, because inert gases are not capable of forming ionic, hydrogen, or covalent bonds with other atoms under physiological conditions. Nevertheless, the receptor hypothesis for general anesthesia mechanisms has recently flourished (4–6). Also, the discovery of endogenous opioids and opioid receptors inspired an expectation (7) that anesthetics may affect either opioid output, opioid binding, or both [see critical reviews by Yakksh and Howe (8) and Ueda and Kamaya (9)].

The only exception to this controversy concerning the anesthetic action site appears to be the idea that the final messenger of anesthetic action is a protein. Still being debated, however, is whether the anesthetic action upon proteins is direct or indirect. Lipid theories assume that the anesthetic action upon proteins is caused by the change in physical properties of lipid membranes where the proteins are embedded (see, for example, Refs. 10–14). Conversely, direct interaction of inhalation anesthetics with water-soluble proteins also has been demonstrated (15–20).

We postulate that the action of inhalation anesthetics is nonspecific, and all macromolecular structures, irrespective of lipid membranes or proteins, are equally affected. All classic and modern potent anesthetics, such as diethylether, chloroform, halothane, methoxyflurane, enflurane, isoflurane, etc., are polar hydrophobic molecules, hence amphipathic, except nitrous oxide. (The action site of apolar anesthetics, such as nitrous oxide, xenon, nitrogen, etc., which are weaker anesthetics, may not be identical with the potent amphipathic anesthetics.) The amphipathy indicates that these molecules

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preferentially solvate into interfaces between water and macromolecules. Here, the term "macromolecule" is used to mean polymer structures including proteins, micelles, lipid membranes, colloids, and all other large structures. We have accumulated experimental evidence showing that these anesthetic molecules preferentially bind to the interface in surfactant micelles and phospholipid membranes.

Biological structures usually contain ionizable moieties and interact electrostatically with water dipoles. The macromolecular structures, such as membranes and proteins, are supported by the hydrogen-bonded water network. Anything that weakens the interaction would disorder the structure, and we hypothesize that the primary action of amphipathic anesthetics is to weaken interactions between water and marcomolecules. The present study is aimed at elucidation of nonspecific anesthetic action upon the surface electrostatic property of a water-soluble protein.

Because surface electrostatic charges interact with the bulk hydrogen ion, the microscopic interfacial pH differs from the macroscopic bulk pH. The surface charge can be estimated from the difference between the interfacial pH, measured by the color of the surface-bound pH-indicator dyes and the bulk pH, measured by a glass electrode. A number of pH-indicator dyes and fluorophores have been used successfully to probe the surface potential of macromolecules. Because of the large number of reports, only a few are arbitrarily mentioned in the References (21–33). The theory and the limitations of this procedure are discussed in the Theory section.

Materials and Methods

Lyophilized crystalline bovine serum albumin, which was delipidated (lipid content less than 0.005%) according to the method of Chen (34), was obtained from Sigma Chemical Co. (St. Louis, MO). Bromothymol blue was obtained from J. T. Baker Chemcial Co. (Phillipsburg, NJ). Methoxyflurane (2,2-dichloro-1,1-difluoroethylmethyl-ether) and enflurane (2-chloro-1,1,2-trifluoroethyl-difluoromethylether) were gifts from Abbott Labs (North Chicago, IL) and Ohio Medical Products (Madison, WI), respectively. Water was purified by distillation followed by treatment with activated charcoal and ion-exchanger columns, and then ultrafiltration in a Millipore system (Bedford, MA). The absence of surface active impurities was ascertained by the dynamic surface tension measurement with a Cahn electrobalance as previously reported (35). The change in surface tension, 10 min after a 10:1 compression of the surface, was confirmed to be less than 0.1 mN/m. The resistivity of the water was maintained at above 16 Mohm·cm. All other chemicals were reagent grade.

The anesthetics were vaporized in a copper kettle of an anesthesia machine and diluted with nitrogen gas. The concentration of the anesthetics was estimated from the ratio of the flows between the saturated vapor and the diluent gas and was confirmed by a Shimadzu gas chromatograph (Columbia, MA). The anesthetic vapor was passed through the gas phase over the albumin solution in an Erlenmeyer flask, maintained in a water bath at 25°, and equilibrated with the solution by slow mixing with a magnetic stirrer. Vigorous mixing or bubbling into the solution was avoided to prevent polymerization and denaturation of the protein at the bubble surface. The maximum effects were obtained in less than 30 min. The concentration of anesthetics in the solution was expressed by the partial pressure of the anesthetics equilibrated with the solution.

Albumin and bromothymol blue were dissolved in freshly prepared water in a concentration of 0.04 mM each. The bulk pH was measured by an Orion 701 pH meter and a combination glass electrode. The absorbance was measured by a Beckman 5270 UV-visible spectrophotometer with a stoppered 1.00-cm light-path cuvette and scanned between 350 nm and 700 nm. The cuvette temperature was maintained at 25° by circulating water from a constant temperature water bath.

Theory

The color and, therefore, the dissociation constant, pK_a, of a weak electrolyte adsorbed on a macromolecular surface differs from that in the bulk water, partly due to the difference in the physical properties of the microenvironment where the probe molecule resides, and partly due to the electrostatic interaction of the surface charge with the dye. According to Boltzmann's law, the discussion constant of the surface-bound dye, pK_a(surface), is expressed

$$pK_a(\text{surface}) = pK_a^I + ze\,\psi/2.3kT \quad (1)$$

where pK_a^I is the intrinsic pK_a of the dye, z is the valence of the electrolyte dye, e is the unit electronic charge, ψ is the surface potential of the dye binding site, k is the Boltzmann constant, and T is the absolute temperature. The pK_a^I includes total nonelectrostatic terms that are mainly solvent properties of the dye binding site. The absolute values for the electrostatic and nonelectrostatic terms are not usually available. But from the difference in the measurable apparent dissociation constants of the free and bound dye, pk_a^F and pK_a^B, respectively, the surface potential of the bound region can be estimated (21–23)

$$\Delta pK_a = pK_a^B - pK_a^F = pK_a^I - pK_a^F + \Delta\psi\, ze/2.3kT \quad (2)$$

The present experiment was performed with a pH-indicator dye, bromothymol blue (BTB), adsorbed on the surface of bovine serum albumin (albumin). The apparent pK_a of this weak electrolyte adsorbed on the protein was measured by the color of the adsorbed dye and the bulk pH, according to the Henderson-Hasselbalch equation

$$pK_a^B = pH + \log([BTB^-]/[BTB^{--}]) \quad (3)$$

where square brackets indicate the concentration and pK_a^B is the observed pK_a of the surface-bound dye. The color of the adsorbed dye is the ratio between acid and base forms, expressed as $[BTB^-]/[BTB^{--}]$, where BTB^- (acid form) is yellow and BTB^{--} (base form) is blue. The two forms are estimated quantitatively by spectrophotometry from the absorbance in the form of:

$$[BTB^-]/[BTB^{--}] = [A_B - A]/[A - A_A] \quad (4)$$

where A is the observed absorbance of the solution, and subscripts A and B signify acid and base forms, respectively. A_A and A_B were measured in the absence of albumin at pH 4.0 and 10.0, respectively, at 615 nm.

The application of this equation is limited to a dye that exclusively binds to proteins with negligible free dye remaining in the bulk aqueous phase. When a significant amount of the dye remains in the aqueous phase, the $[BTB^-]/[BTB^{--}]$ term must be corrected by the partition coefficient of the dye between free and bound forms. In the case of bromothymol blue and bovine serum albumin, we have previously established by an ultrafiltration study (36) that practically all dye molecules are bound to the protein even in the presence of anesthetics in the concentration range at which the present experiment is performed.

The binding parameters of bromothymol blue to bovine serum albumin have been estimated from the Scatchard plot at

several temperatures (36). At 20°, the high affinity binding showed $K = 3.10 \cdot 10^6$ M^{-1} and $n = 1.9$, and the low affinity binding showed $K = 8.22 \cdot 10^3$ M^{-1} and $n = 32$, where K is the affinity constant and n is the number of binding sites. From the temperature dependence of the affinity constant, the enthalpy changes of the high and low affinity bindings were estimated to be −1.88 and −1.62 kcal·mol^{-1}, respectively. The entropy changes were 23.3 and 12.4 cal·mol^{-1}·K^{-1}, respectively. The higher affinity is mainly attributable to the entropy change. A statistical mechanical analysis revealed that the Scatchard plot cannot be applied in the presence of low anesthetic concentrations (36). At high anesthetic concentrations, however, the dye binding approaches asymptotically to a limiting value and the apparent association constant (36) can be renormalized to estimate the intrinsic association constant, K, and n. With methoxyflurane at $1.0 \cdot 10^{-2}$ atm, which is about one order of magnitude higher than the clinical range, the affinity constants were decreased in the high and low affinity bindings without measurable effect upon the number of binding sites (36).

Results and Discussion

Fig. 1 shows typical scanning data on the response to the bulk pH change of the color of bromothymol blue adsorbed on albumin. These studies were performed under salt-free conditions to avoid the change in the albumin surface charge by counter-ion binding. Except at pH 2.3 and 11.73, a well defined isosbestic point was observed at 485 nm. The anomaly observed with the low pH scan appears to be caused by the acid denaturation that occurs below pH 3.5 and the N-F transition (conformational change) of bovine serum albumin that occurs between pH 3.5 and pH 4.5. For this reason, the extremely low pH region was excluded from the study. Bovine serum albumin undergoes irreversible alkaline denaturation at about pH 10.5, and this region was also excluded. The ratio between acid and base forms of bromothymol blue was estimated from Eq. 4 between pH 4.5 and 10, and log([BTB^{--}]/[BTB$^-$]) was plotted against the bulk pH value in Fig. 2. As shown in this figure, the data scatter was negligible. The log([BTB^{--}]/[BTB$^-$])-pH diagram was characterized by a linear relationship with a slight

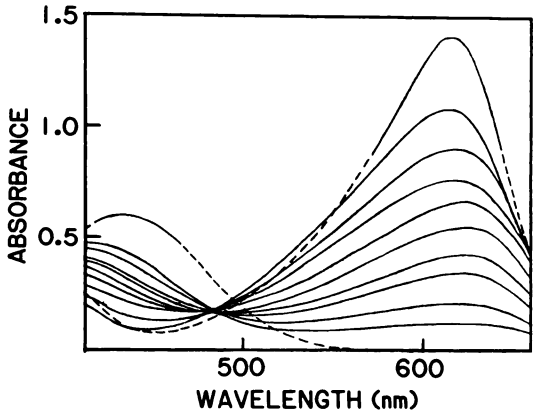

Fig. 1. UV-visible spectra of bromothymol blue adsorbed on bovine serum albumin. The bulk pH values are (from the *top*) at 610 nm: 11.73, 10.42, 9.57, 8.74, 7.40, 6.93, 6.47, 6.16, 5.45, 4.98, and 2.3. Except at pH 11.73 and 2.3, a well defined isosbestic point is observed at 485 nm. The anomalies in extremely high and low pH values are probably caused by albumin denaturation.

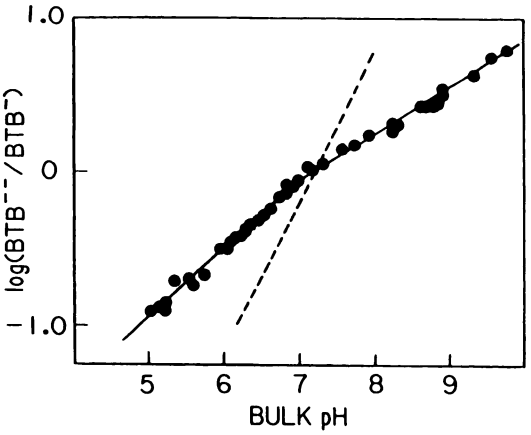

Fig. 2. The change in color of bromothymol blue, expressed by log([BTB^{--}]/[BTB$^-$], adsorbed on bovine serum as a function of bulk pH. The titration of bromothymol blue in the absence of albumin is also shown by the *dashed line* for comparison.

bend at about pH 7, which was presumably caused by the N-B transition (conformational change) of bovine serum albumin that occurs between pH 7 and 9. For comparison, the pH-induced color change of bromothymol blue in the absence of bovine serum albumin is also shown in Fig. 2.

Katchalsky and Spitnik (37) reported that the dissociation of ionizable moiety in a macromolecule (polymethacrylic acid) is different from the dissocation of small ionizable molecules, and they proposed the generalized Henderson-Hasselbalch equation, in which the relationship between the bulk pH and the dissociation constant is described by a linear relationship, using a parameter, n, as follows:

$$\text{pH} = \text{pK}_a + n \log([\text{base}]/[\text{acid}]) \quad (5)$$

When n is unity, this reduces to the conventional Henderson-Hasselbalch equation. The present data yield $n = 3.2$ for the alkaline region and $n = 2.1$ for the acid region. In this treatment, the parameter n includes all interaction forces between the ionizable moiety and neighboring moieties, and can probably be subdivided into the electrostatic and nonelectrostatic parameters described previously.

The color change of the bound dye in response to the bulk pH change is caused mainly by the change in the pH of the microscopic region where the dye molecules reside. But because the true pK_a value of the bound dye is not known, and because the present study is concerned with the relative change in the physical properties of albumin surface by the anesthetic interaction, the color change of the dye is expressed by the apparent pK_a^B value of the dye, estimated according to Eq. 3, and is shown in Fig. 3. The apparent pK_a^B of the bound dye shown in this figure is unlike the usual bulk pK_a values of small molecules, which are invariant to pH, and the value was a function of the bulk pH. This is because the changes in the albumin surface properties, caused by the change in the bulk pH, are squeezed into this apparent surface pK_a^B value.

The electrostatic effect of local surface charges on pK_a^B was screened by increasing the ionic strength of the solution. Addition of NaCl to a final concentration of 1.0 M blocked the surface electrostatic forces by forming an ion-pair and increased the log([BTB$^-$]/[BTB^{--}]) value. The observed pK_a^B was 7.91 at bulk pH 7.00. This pK_a^B represents the contribution from the nonelectrostatic term to the apparent dissociation constant

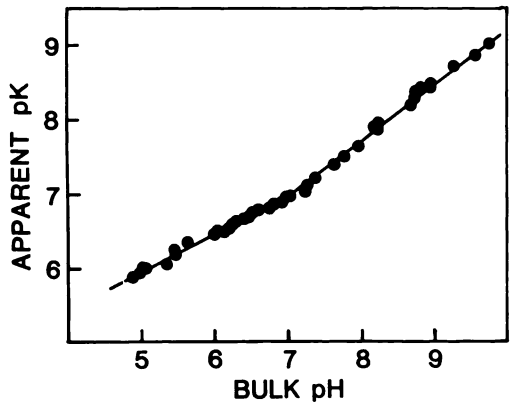

Fig. 3. Apparent pK_a value of bromothymol blue adsorbed on bovine serum albumin as a function of bulk pH. Because the change in the color of bromothymol blue caused by the difference in the local pH and the bulk as well as the difference in the physical property between the two domains is squeezed into the pK_a of the dye, the value varies with pH.

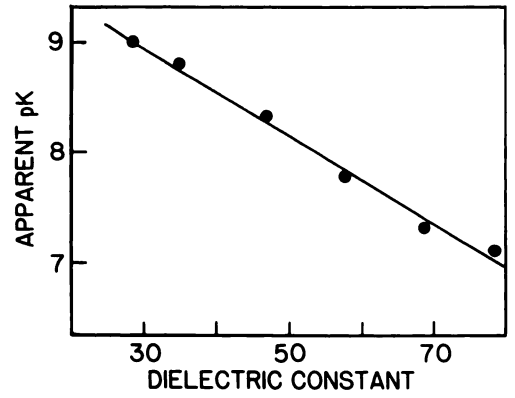

Fig. 4. The change in the color of bromothymol blue expressed by apparent pK_a values according to Eq. 3 as a function of solvent dielectric constant. The solvent dielectric constant was varied by mixing water and ethanol.

and nearly equals the intrinsic pK_a' expressed in Eq. 1. This nonelectrostatic effect upon the electronic spectra of the dye consists of the solvent effect of the binding site and apparently involves multiple factors, such as charge transfer (short range), energy transfer (long range), hydrogen bonding, dipole interaction, etc., between dye molecules and the binding site.

For comparison, the effect of the solvent property upon the color of bromothymol blue was investigated in ethanol/water mixtures. The apparent pK_a of bromothymol blue dissolved in solvents with various ethanol/water ratios showed a linear relationship to the solvent dielectric constant (Fig. 4). When interpolated, the apparent dissociation constant of bromothymol blue adsorbed on the albumin surface, whose surface charges are screened by 1 M NaCl, was about equivalent to that in the dielectric constant of 55 of the ethanol/water mixture. This does not necessarily mean that the dielectric constant of the dye binding site is exactly 55, because the property of the albumin surface is not identical to the ethanol/water mixture. Usually, a change in electronic spectra of a molecule in an organic solvent/water mixture is a linear function of the dielectric constant of the solution, especially in a homologous series of organic solvents (38, 39). In organic solvents with diverse structures, however, dyes occasionally show different electronic spectra:dielectric constant relationships (38, 39). Nevertheless, the present result indicates that a decrease in the dielectric constant increases the apparent pK_a of bromothymol blue ($[BTB^-/BTB^{--}]$ ratio increases): the surface area is more hydrophobic than the bulk water. For anionic dyes, such as bromothymol blue, a decrease in the solvent dielectric constant has an effect like adding acid to the environment.

Under the condition of high salt screening of electrostatic effects, anesthetic action upon the apparent dissociation constant of the bound dye was measured at 1.0 M NaCl and 0.1 M phosphate buffer, pH 7.0, and is shown in Fig. 5. The standard deviation of each data point was within the size of the symbol. The effects of methoxyflurane and enflurane on the apparent pK_a' value were slight increases in the low anesthetic concentration ranges and slight decreases in the high concentration ranges. The increase in the apparent pK_a values at low anesthetic concentration is probably caused by the decrease of the dielectric constant of the dye binding site. The decrease in the apparent pK_a values at higher anesthetic concentrations may be caused by dissociation of the dye from the binding site. The concentration ranges, where the apparent pK_a values started to decrease, coincide with those of our previous ultrafiltration study (36) on the dye binding, where the dye molecules started to dissociate from the binding site.

As stated earlier, the anesthetic action between electrostatic and nonelectrostatic terms is difficult to separate. However, from the present result, shown in Fig. 5, the anesthetic action on the nonelectrostatic parameter can safely be neglected. Then, according to Eq. 2, the anesthetic effect upon the apparent dissociation constant is expressed as

$$\Delta pK_a^B = pK_a^{BA} - pK_a^B = ze\Delta\Delta\psi/2.303kT \qquad (6)$$

or

$$\Delta\Delta\psi = 2.303\ \Delta pK_a^B kT/ze \qquad (7)$$

where pK_a^{BA} and pK_a^B are the apparent dissociation constants of bromothymol blue adsorbed on the albumin surface in the presence and absence of anesthetics, respectively, and $\Delta\Delta\psi$ is the change in the surface potential caused by anesthetics.

Fig. 6 depicts the anesthetic effects on the surface potential, $\Delta\Delta\psi$ calculated from Eq. 7. The order of the decrease in the surface potential followed that of the anesthetic potencies. The partial pressures of methoxyflurane, enflurane, and diethylether which decreased the surface potential 10 mV were 0.17·

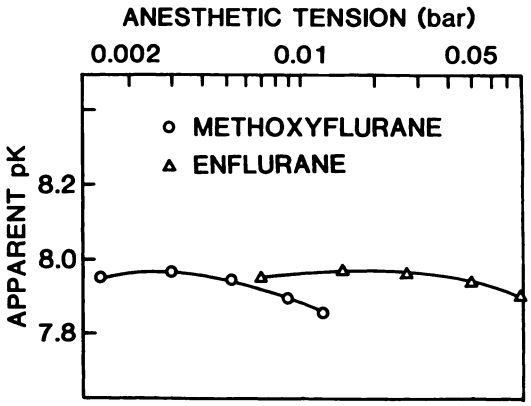

Fig. 5. Anesthetic effects upon the apparent pK_a value of bromothymol blue, bound on bovine serum albumin, under the condition of high salt shielding of electrostatic effect by 1.0 M NaCl.

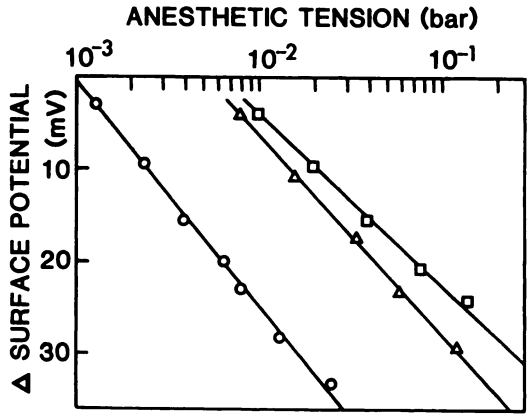

Fig. 6. The surface potential change in bovine serum albumin by methoxyflurane (○), enflurane (△), and diethylether (□).

10^{-2}, $1.7 \cdot 10^{-2}$, and $2.1 \cdot 10^{-2}$ bar, respectively. These values are in excellent agreement with the clinical potencies of these anesthetics (40).

The reversibility of the effects of the anesthetics upon the surface potential was determined by washing out the anesthetics from the albumin solution by nitrogen gas. The surface potential value returned to the control after a 1-hr washout of the anesthetic gases. The pK_a of bromothymol blue in aqueous solution in the absence of albumin was not affected by the presence of the anesthetics.

The addition of methoxyflurane in the presence of albumin shifted the wavelength of the absorbance maximum slightly to the longer wavelength. At its highest partial pressure ($2.5 \cdot 10^{-2}$ bar), the red shift by methoxyflurane was 4 nm, which amounted to about 0.7% of the wavelength of the absorbance maximum (615 nm). Due to this red shift, the effect of methoxyflurane upon pK_a^B was slightly underestimated. Diethylether and enflurane did not change the wavelength.

The present result is in contrast to the report by Koh *et al.* (30), who used 1-dimethylaminonaphthalene-5-sulfonyl chloride (DANSYL chloride), covalently bound to bovine serum albumin, and found that the change in the apparent pK_a of the dye by inhalation anesthetics did not correlate well to their clinical potencies. They attributed this failure in correlating with clinical potency to the position of the dye binding site, which is a hydrophobic and relatively internal area of the protein. It was suggested (30) that the change in the protein internal structure may not be directly related to the anesthesia mechanisms.

The cause of the decrease in surface potential is not readily available. A conformational change of albumin by anesthetic interaction is always a possibility. A decrease in the surface charge density by protein conformational change would shift the surface potential toward zero. Another possibility is the change in the counterion binding. An increase in counterion binding to micelle surfaces by anesthetic adsorption (enflurane) has recently been observed by ^{23}Na nuclear magnetic resonance spectroscopy (41). Although elucidation of the exact cause of the anesthetic effect requires further investigation, a decrease in the surface potential of bovine serum albumin by clinical concentrations of anesthetics is obvious. A decrease in the surface potential would decrease the electrostatic force imposed upon the water dipole, and is expected to dissolute the electrostricted water structures clustered around the surface charges. Because the volume of electrostricted water structure is smaller than that of bulk water (42), release of electrostricted water molecules increases the total volume. This mechanism would contribute to the excess volume increase associated with anesthetic interactions with macromolecular structures (43).

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References

1. Cullen, S., and E. Gross. The anesthetic properties of xenon in animals and human beings, with additional observations on krypton. *Science (Wash. D.C.)* **113**:580–582 (1951).
2. Butler, T. Theories of general anesthesia. *Pharmacol. Rev.* **2**:121–160 (1950).
3. Featherstone, R. M., and C. A. Muehlbaecher. The current role of inert gases in the search for anesthesia mechanisms. *Pharmacol. Rev.* **15**:97–121 (1963).
4. Richards, C. D. The action of anesthetics on synaptic transmission. *Gen. Pharmacol.* **9**:287–293 (1978).
5. Franks, N. P., and W. R. Lieb. Is membrane expansion relevant to anaesthesia? *Nature (Lond.)* **292**:248–251 (1981).
6. Smith, E. B., F. Bowser-Riley, S. Daniels, I. T. Dunbar, C. B. Harrison, and W. D. M. Paton. Species variation and the mechanism of pressure-anaesthetic interactions. *Nature (Lond.)* **311**:56–57 (1984).
7. LaBella, F. S. Is there a general anesthesia receptor? *Can. J. Physiol. Pharmacol.* **59**:432–442 (1981).
8. Yakksh, T. L., and J. R. Howe. Opiate receptors and their definition by antagonists. *Anesthesiology* **56**:246–249 (1982).
9. Ueda, I., and H. Kamaya. Molecular mechanisms of anesthesia. *Anesth. Analg.* **63**:929–945 (1984).
10. Seeman, P. The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* **24**:583–655 (1972).
11. Trudell, J. R. A unitary theory of anesthesia based on lateral phase separations in nerve membranes. *Anesthesiology* **46**:327–332 (1977).
12. Miller, K. W., W. D. M. Paton, R. A. Smith, and E. B. Smith. The pressure reversal of general anesthesia and the critical volume hypothesis. *Mol. Pharmacol.* **9**:131–143 (1973).
13. Tsong, T. Y., M. Greenberg, and M. I. Kanehisa. Anesthetic action on membrane lipids. *Biochemistry* **16**:3115–3121 (1977).
14. Simon, S. A., T. J. McIntosh, P. B. Bennett, and B. B. Shrivastav. Interaction of halothane with lipid bilayers. *Mol. Pharmacol.* **16**:163–170 (1979).
15. Ueda, I. Effects of diethylether and halothane on firefly luciferin bioluminescence. *Anesthesiology* **26**:603–606 (1965).
16. Adey, G., E. Wardley-Smith, and D. White. Mechanism of inhibition of bacterial luciferase by anesthetics. *Life. Sci.* **17**:1849–54 (1975).
17. Ueda, I., and W. Mietani. Microsomal ATPase of rabbit brain and effects of general anesthetics. *Biochem. Pharmacol.* **16**:1370–1374 (1967).
18. Shoenborn, B. P. Binding of anesthetics to protein: An X-ray crystallographic investigation. *Fed. Proc.* **27**:888–894 (1968).
19. Laasberg, L. H., and J. Headley-White. Optical rotatory dispersion of hemoglobin and polypeptides. Effect of halothane. *J. Biol. Chem.* **246**:4886–4893 (1971).
20. Shibata, A., H. Kamaya, and I. Ueda. Electrostriction around colloid molecules and interfacial action of inhalation anesthetic: volume function. *J. Colloid Interface Sci.* **90**:487–494 (1982).
21. Hartley, G. S., and J. W. Roe. Ionic concentrations at interfaces. *Trans. Faraday Soc.* **36**:101–109 (1940).
22. Mukerjee, P., and K. Banerjee. A study of the surface pH of micelles using solubilized indicator dyes. *J. Phys. Chem.* **68**:3567–3574 (1964).
23. Moller, J. V., and U. Kragh-Hansen. Indicator dyes as probes of electrostatic potential changes on macromolecular surfaces. *Biochemistry* **14**:2317–2323 (1975).
24. Bakker, E. P., J. C. Arents, J. P. M. Hoebe, and H. Terada. Surface potential and the interaction of weakly acidic uncouplers of oxidative phosphorylation with liposomes and mitochondria. *Biochim. Biophys. Acta* **387**:491–506 (1975).
25. Fernandez, M. S., and P. Fromherz. Lipoid pH indicators as probes of electrical potential and polarity in micelles. *J. Phys. Chem.* **81**:1755–1761 (1977).
26. Funasaki, N. The dissociation constants of acid-base indicators on the micellar surface of dodecyldimethylamine oxide. *J. Colloid Interface Sci.* **60**:54–59 (1977).
27. Vaz, W. L. C., A. Nicksch, and F. Jahnig. Electrostatic interactions at charged lipid membranes. Measurement of surface pH with fluorescent lipoid pH indicators. *Eur. J. Biochem.* **83**:299–305 (1978).
28. Mashimo, T., I. Ueda, D. D. Shieh, H. Kamaya, and H. Eyring. Hydrophilic region of lecithin membranes studied by bromothymol blue and effects of an inhalation anesthetic, enflurane. *Proc. Natl. Acad. Sci. USA* **76**:5114–5118 (1979).
29. Okuda, C., and K. Ogli. Fluorometric study with 1-anilino-8-naphthalene sulfonate on membrane surface charge changes induced by inhalation anesthetics. *FEBS Lett.* **101**:399–402 (1979).
30. Koh, J., C. Tashiro, H. Ikuta, J. Koga, and N. Kuroki. The effect of volatile anesthetics upon acid-base equilibria of ionizable ligand bound to protein

molecules. *J. Jpn. Anesth. Assoc.* **29**:671–676 (1980).
31. Chiu, V. C. K., D. Mouring, B. D. Watson, and D. H. Heynes. Measurement of surface potential and surface charge densities of sarcoplasmic reticulum membranes. *J. Membr. Biol.* **56**:121–132 (1980).
32. Nakagaki, M., I. Katoh, and T. Handa. Surface potential of lipid membrane estimated from the partitioning of methylene blue into liposome. *Biochemistry* **20**:2208–2212 (1981).
33. Tanabe, H., N. Kamo, and Y. Kobatake. Fluorometric estimation of surface potential change associated with chemotactic stimulation in Tetrahymena pyriformis. *Biochim. Biophys. Acta* **805**:345–353 (1984).
34. Chen, R. F. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **242**:173–181 (1967).
35. Shibata, A., Y. Suezaki, H. Kamaya, and I. Ueda. Adsorption of inhalation anesthetics on the air/water interface and the effect of water structure. *Biochim. Biophys. Acta* **646**:126–134 (1981).
36. Mashimo, T., Y. Suezaki, and I. Ueda. Anesthetic-protein interaction: suppression of dye binding and limitation of applicability of the Scatchard plot. *Physiol. Chem. Phys.* **14**:543–551 (1982).
37. Katchalsky, A., and P. Spitnik. Potentiometric titration of polymethacrylic acid. *J. Polym. Sci.* **2**:432–446 (1947).
38. Kaneshina, S., H. Kamaya, and I. Ueda. Benzyl alcohol penetration into micelles, dielectric constant of the binding site, partition coefficient and high pressure squeeze-out. *Biochim. Biophys. Acta* **777**:75–83 (1984).
39. Rehfeld, S. J. Solubilization of benzene in aqueous solutions of n-alkylammonium bromide, n-alkanols, acetic acid, and perchloric acid measured by differential spectroscopy. *J. Am. Chem. Soc.* **95**:4489–4492 (1973).
40. Eger, E. I., II. *Anesthetic Uptake and Action.* Williams & Wilkins, Baltimore, 82 (1976).
41. Yoshida, T., K. Taga, H. Okabayashi, K. Matsushita, H. Kamaya, and I. Ueda. Counterion binding to micelles measured by sodium-23 NMR relaxation times: enhancement by anesthetics. *J. Colloid Interface Sci.*, in press.
42. Kauzmann, W. Some factors in the interpretation of protein denaturation. *Adv. Protein Chem.* **14**:1–63 (1959).
43. Mori, T., N. Matubayasi, and I. Ueda. Membrane expansion and inhalation anesthetics: mean excess volume hypothesis. *Mol. Pharmacol.* **25**:123–130 (1984).

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