[Chem. Pharm. Bull. 34(10)4301—4307(1986)]

Erythrocyte Membrane Penetration of Basic Drugs and Relationship between Drug Penetration and Hemolysis

TARO OGISO,* MASAHIRO IWAKI, MISA KIMORI and CHIHO TSUKAWAKI

Faculty of Pharmaceutical Sciences, Kinki University, Kowakae 3–4–1, Higashi-Osaka 577, Japan

(Received April 14, 1986)

To clarify the relationship between hemolytic activity and membrane penetration of drugs, some tranquilizers and antihistaminics were compared with each other as regards the amount of drugs able to penetrate human erythrocyte membrane at 27 and 37 °C, and the activation energy for penetration was calculated. The drugs exerting strong hemolytic action penetrated the membrane much more effectively than those with less activity. The relation between the amount (A) of drug penetrated *vs.* extracellular drug concentration (C) could be generally described by a linear equation: $\log A$ (10^{-8} mol) $= 0.817 \cdot \log C$ (10^{-4} M) $+ b$. With 8 phenothiazines, the correlation coefficient between A and the value of $1/C_1$ or $1/C_{50}$ (C_1 is the initial concentration inducing hemolysis and C_{50} is the concentration inducing 50% hemolysis) was 0.916 ($p < 0.01$) or 0.927 ($p < 0.01$), respectively. The amount of drugs penetrated at the C_{50} was $(0.7—4.1) \times 10^{-17}$ mol/ghost and $(1.7—10.6) \times 10^{-2}$ mol/mol phospholipid. The drug penetration into the membrane could be approximately described by the Freundlich equation, but not the Scatchard equation. The activation energy for drug penetration was in the range from 1.07 to 6.16 kcal, and there was no clear-cut relationship between the energy and the above parameters.

Keywords——basic drug; human erythrocyte membrane; membrane penetration; hemolytic activity; penetration activation energy; Freundlich equation; hemolysis–penetration correlation

Many kinds of drugs, such as tranquilizers, antihistaminics and anesthetics, stabilize erythrocytes against hypotonic hemolysis at low concentrations,[1-12] but these drugs cause lysis at higher concentrations.[8,12-15] The mechanism of erythrocyte hemolysis at higher drug concentrations has been studied by some investigators[14,16] including us.[17,18] Previously, we found that tranquilizers and antihistaminics have different effects on the rate and extent of hemolysis.[15] We also found that a major factor in the induction of hemolysis is the amount of drugs penetrated into the membrane and other factors are increased fluidity of membrane lipids and large molecular volume of the drugs penetrated.[19]

The purpose of this study was to try to relate the penetration of basic drugs to the membrane actions. In order to clarify the relationship between hemolytic activity and membrane penetration of drugs, some tranquilizers and antihistaminics were compared with each other as regards the amount of drugs penetrated into the membrane, the effect of temperature on the penetration and the activation energy for penetration. It was found that the amount of drug penetrated could be approximately described by the Freundlich equation.

Experimental

Drugs——Promazine hydrochloride (Hokuriku Pharmaceutical Co., Fukui), promethazine hydrochloride, prochlorperazine methanesulfonate, levomepromazine hydrochloride (Shionogi Pharmaceutical Co., Osaka), tri-

meprazine tartrate (Dai-ichi Pharmaceutical Co., Tokyo), perazine dimalonate (Morishita Pharmaceutical Co., Osaka), chlorpromazine hydrochloride, prothipendyl hydrochloride (Nippon Shinyaku, Kyoto), and carpipramine hydrochloride (Yoshitomi Pharmaceutical Co., Osaka) were used throughout this experiment. The other reagents were of special or analytical grade.

Preparation of Hemoglobin-Free Erythrocyte Ghosts——Citrate–phosphate–dextrose (CPD) blood, obtained from healthy adult donors, was washed three times with isotonic NaCl–phosphate buffer (NaCl 90.0 g, $NaH_2PO_4 \cdot 2H_2O$ 3.43 g and $Na_2HPO_4 \cdot 12H_2O$ 34.425 g/10 l, pH 7.4) and the erythrocyte ghosts were prepared by the method of Dodge *et al.*[20]

Measurement of Drug Penetrated into the Membrane——A ghost suspension (1 ml, 5 mg protein/ml) was added to 10 ml of drug solution in isotonic buffer at various concentrations, and the mixture was incubated for 30 min at 37 or 27 °C. The membrane was carefully but quickly washed twice with 20 ml of isotonic buffer. After centrifugation, 4 ml of 0.01 N NaOH was added to the ghost pellet and the whole was mixed vigorously for 1 min. The suspension was extracted with 15 ml of water-saturated ethyl acetate in a 40 ml centrifuge tube by shaking for 15 min, and the two layers were separated by centrifugation. The drug contained in 10 ml of the ethyl acetate layer was extracted into 5 ml of 0.1 N HCl by shaking followed by centrifugation. The aqueous layer was carefully and as completely as possible transferred to another tube. The remaining ethyl acetate layer was again extracted with 5 ml of 0.1 N HCl. Subsequently, the drug concentration in both aqueous layers was spectrophotometrically determined at the extinction maximum of the drug. Since the drug extraction ratio by this method was different for each drug, the drug concentration was corrected by using the extraction ratio obtained after addition of a definite amount of the drug under the same conditions.

Measurement of Activation Energy for Drug Penetration——The activation energy (ΔE) was calculated from the amounts of drug penetrated at 37 and 27 °C according to the Arrhenius equation:

$$\Delta E = \frac{2.303 \cdot \boldsymbol{R} \cdot T_1 \cdot T_2}{T_1 - T_2} \log \frac{k_1}{k_2}$$

where T_1 and T_2 are the absolute temperatures, k_1 and k_2 are the amounts of drug penetrated at 37 and 27 °C, respectively and $\boldsymbol{R}$ is the gas constant.

Protein Determination——Protein concentration was determined by the procedure described by Lowry *et al.*[21] with bovine albumin, fraction V, as a standard.

Data Analysis——The calculation and statistical analyses were carried out with the aid of an NEC PC-9800 personal computer. Correlation analyses were done by the least-squares linear regression method. Correlation coefficients were examined for significance ($p < 0.05$) by means of the t-test.

Results

Amount of Drugs Penetrated into Erythrocyte Membrane and Regression Analysis of the Penetration Curve

Figure 1 shows the amount of drug penetrated into the erythrocyte membrane as a function of initial drug concentration. Logarithmic plots of the amount of drug penetrated *vs.* extracellular drug concentration were approximately linear over the range of concentrations tested, although there was slight discrepancy for some drugs at higher concentrations. This suggests that the amount of drug penetrated increases exponentially in parallel with the drug concentration in the extracellular medium, with characteristic differences in the penetration profile for each drug. Prochlorperazine and chlorpromazine were highly partitioned into the cell membrane, while promethazine was less penetrative at the same concentration.

The relationship between the amount (A) of drug penetrated and the extracellular drug concentration (C) could be described by a linear equation defining a geometric curve. The regression equation of the relationship was: $\log A$ (10^{-8} mol/ghost ml) $= a \cdot \log C$ (10^{-4} M) $+ b$ where a and b are a slope and an intercept, respectively. The value of a and b for each drug is shown in Table I with the r value. The value of a estimated ranged from 0.669 to 1.098. The mean value of slope a with 95% confidence intervals was 0.817 ± 0.149. Thus, the regression equation for the drugs tested could be described as follows: $\log A = 0.817 \cdot \log C + b$.

Relationship between Amount of Drug Penetrated into the Membrane and Hemolytic Concentration

In order to confirm our previous proposal that the amount of drug penetrated into the

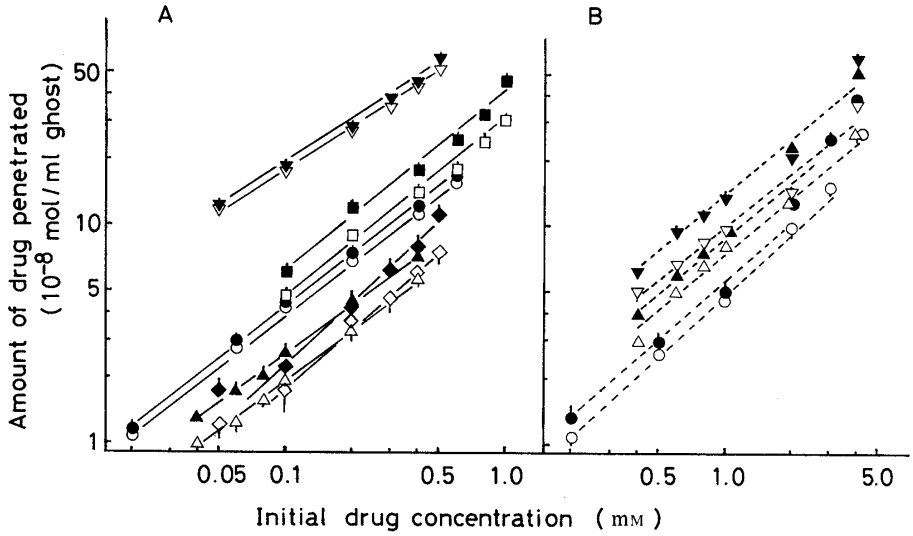

Fig. 1. Relationship between Amount of Drug Penetrated into Erythrocyte Membrane and Initial Drug Concentration, and the Effect of Temperature on Drug Penetration

Closed and open symbols show the amounts of drug penetrated at 37 and 27 °C, respectively. Each point represents the mean ± S.D. of 4—6 experiments. A: chlorpromazine (■, □), prochlorperazine (▼, ▽), perazine (●, ○), levomepromazine (▲, △), carpipramine (◆, ◇). B: promazine (▼, ▽), promethazine (●, ○), trimeprazine (▲, △).

TABLE I. Slope (a) and Intercept (b) Values in the Regression Equation[a] of the Relationship between Amount of Drug Penetrated and Initial Drug Concentration

Drug	Temp. (°C)	a	b	r
Chlorpromazine	27	0.677	5.794	0.994[b]
	37	0.764	6.763	0.989[b]
Promethazine	27	0.968	0.528	0.999[b]
	37	1.068	0.544	0.989[c]
Carpipramine	27	0.902	2.159	0.998[b]
	37	0.975	1.750	0.997[b]
Levomepromazine	27	0.770	1.906	0.999[b]
	37	0.747	2.546	0.998[b]
Promazine	27	0.647	2.093	0.997[b]
	37	0.723	2.426	0.990[c]
Prochlorperazine	27	0.618	18.388	0.999[b]
	37	0.669	18.684	0.997[b]
Perazine	27	0.771	4.014	0.999[b]
	37	0.786	4.227	0.999[b]
Trimeprazine	27	0.882	0.973	0.985[c]
	37	1.098	0.881	0.996[b]

a) The regression equation is: $\log A = a \log C + b$. b) $p < 0.001$, c) $p < 0.01$.

membrane is probably a major factor in the induction of hemolysis, the amount of drug penetrated was compared with the hemolytic concentration (C_1 and C_{50}: C_1, the initial concentration inducing hemolysis; C_{50}, the concentration inducing 50% hemolysis) estimated previously.[19] The results for 8 phenothiazines are summarized in Table II. Prochlorperazine and chlorpromazine, which induced hemolysis at lower concentrations, had a high drug level in the membrane, whereas prothipendyl, promethazine and trimeprazine, having lower lytic activities, were less effectively partitioned into the membrane. The correlation between the

TABLE II. Relationship between Amount of Drug Penetrated and Hemolytic Concentration at 37 °C

Drug	C_1 (10^{-4} M)	Drug in membrane (10^{-8} mol/ml ghost)	C_{50} (10^{-4} M)	Drug in membrane (10^{-8} mol/ml ghost)
Chlorpromazine	3.60	17.0	5.40	23.6
Promethazine	10.60	6.5	13.50	8.4
Levomepromazine	7.47	11.1	9.45	—
Promazine	11.70	14.6	13.30	16.3
Prochlorperazine	1.84	27.6	2.39	33.2
Perazine	3.97	12.3	5.56	16.5
Prothipendyl	23.30	4.9	27.00	5.4
Trimeprazine	10.50	9.9	13.80	12.9

C_1, initial drug concentration inducing hemolysis; C_{50}, drug concentration inducing 50% hemolysis; Drug in membrane, the values calculated at C_1 or C_{50}. r (amount of drug and $1/C_1$)=0.916 ($p<0.01$); r (amount of drug and $1/C_{50}$)=0.927 ($p<0.01$).

TABLE III. Amount of Drug per Erythrocyte Membrane and Phospholipid at C_{50} and 37 °C

Drug	Drug in membrane	
	(10^{-17} mol/ghost[a])	(10^{-2} mol/mol phospholipid[a])
Chlorpromazine	2.88	7.54
Promethazine	1.02	2.69
Carpipramine	0.84	2.05
Promazine	1.99	5.21
Prochlorperazine	4.05	10.61
Perazine	2.01	5.27
Prothipendyl	0.66	1.73
Trimeprazine	1.57	4.12

a) These values were calculated based on the result reported by Dodge *et al.*,[20] and the average molecular weight of phospholipid was presumed to be 775.

amount of drug penetrated and $1/C_1$ of $1/C_{50}$ was examined. The calculated correlation coefficients (r) were 0.916 between the amount and $1/C_1$ ($p<0.01$) and 0.927 between the amount and $1/C_{50}$ ($p<0.01$), suggesting that the drugs with high hemolytic activity could penetrate much more effectively into the membrane. Data for carpipramine were excluded from the computation since it is not a phenothiazine.

The amount of drug penetrated into the membrane at C_{50} is expressed as mol per ghost or mol per mol of phospholipid in Table III. It was found that the amount of drug required to disturb the membrane structure was $(0.7—4.1)\times 10^{-17}$ mol/ghost or $(1.7—10.6)\times 10^{-2}$ mol/mol phospholipid.

Effect of Temperature on Drug Penetration and Activation Energy for Drug Penetration

The amont of drug penetrated into the membrane measured at 27 °C is plotted in Fig. 1. The slope of each plot at 27 °C was approximately parallel to that at 37 °C, so that the activation energy was obtained from the slope according to the Arrhenius equation. The calculated activation energies are shown in Table IV, and range from 1.07 to 6.16 kcal. The activation energies for perazine (1.07 kcal) and prochlorperazine (1.21 kcal) were dramatically lower than those for other drugs. However, a clear-cut relationship was not observed between the energy and C_{50} or the amount of drug penetrated.

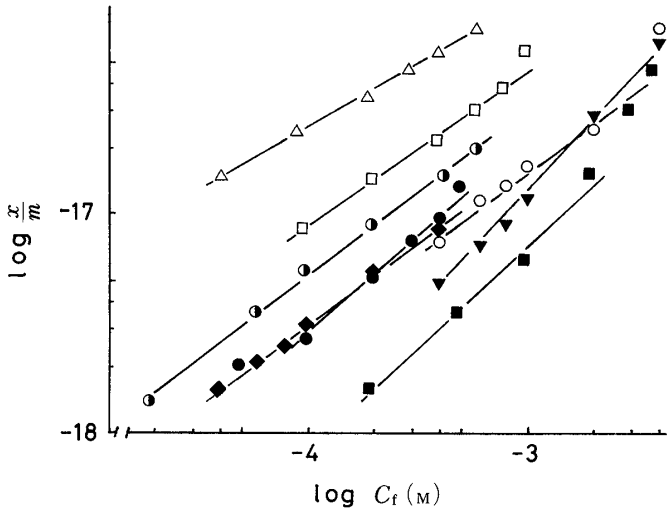

Fig. 2. Freundlich Plots for Drug Penetration into Erythrocyte Membrane

Chlorpromazine (□), promethazine (■), levomepromazine (◆), promazine (○), prochlorperazine (△), perazine (◑), carpipramine (●), trimeprazine (▼).

TABLE IV. Activation Energy for Drug Penetration into the Membrane

Drug	Activation energy (cal)
Chlorpromazine	4447± 973
Promethazine	3125±1333
Carpipramine	5365± 740
Levomepromazine	5534± 629
Promazine	6165± 550
Prochlorperazine	1209± 473
Perazine	1067± 268
Trimeprazine	4424±1335

Freundlich Plots for Drug Penetration into the Membrane

When the amount of drug penetrated into the membrane at 37 °C was plotted according to the Freundlich equation[22] ($\log x/m = 1/n \cdot \log C_f + \log K$, where x is the amount of drug penetrated, m is the number of ghosts, C_f is the drug concentration after equilibrium and n and K are constants), an approximately straight line was obtained for all drugs in the range of concentrations tested, as shown in Fig. 2. This indicates that drug penetration into the erythrocyte membrane can be roughly analyzed by means of the Freundlich equation. When the drug penetration was analyzed according to the Scatchard equation, there was no linear relationship between the amount of drug penetrated and the concentration.

Discussion

The study of the interaction of small amphipaths with membranes is of considerable interest in biology and physiology. Lipid membranes play a crucial role in the phenomenon of local anesthesia.[23] The anesthetic action of a drug depends on its membrane solubility (expressed as a partition coefficient, which is the equilibrium concentration between the lipid and the aqueous phase). Our previous study showed that drugs having strong hemolytic activity were highly partitioned into the cell membrane.[19] To clarify further the relationship between the membrane penetration and the hemolytic action of basic drugs, and to obtain insight into the mode of drug penetration, some experiments were carried out on 9 basic drugs with human erythrocyte membrane.

In the present work, we found that the relationship between the amount (A) of drug penetrated *vs.* extracellular drug concentration (C) could be described by a linear equation defining a geometric curve, $\log A = 0.817 \cdot \log C + b$ (Fig. 1 and Table I). It was therefore shown that the amount of drug penetrated increased exponentially in relation to the extracellular aqueous concentration of drug, and that the drugs exerting strong hemolytic action could generally penetrate into the cell membrane more easily than those with less activity. The good correlation between the amount of drug penetrated and $1/C_1$ or $1/C_{50}$ (Table II) strongly supports this concept. Additionally, this observation strengthens our original conclusion that the amount of drug penetrated into the membrane is probably a

major factor in the induction of lysis.[19]

The number of sites present in the erythrocyte membrane and their affinity for the drug have been determined by plotting the reciprocals of the drug concentrations in the medium and in the membrane.[24] However, up to the present, the drug penetration into the membrane has not been analyzed in greater detail. Our data indicated that the drug penetration into erythrocyte membrane could be approximately analyzed by means of Freundlich plots (Fig. 2).

The amount of drug penetrated into membrane at the C_{50} was $(1.7—10.6) \times 10^{-2}$ mol/mol phospholipid (Table III), suggesting that when one mol of basic drug is present per 9.4—58.8 mol of membrane phospholipid, drastic perturbation of the membrane structure (presumably lipid bilayer structure) should be induced. The classical partition theory of anesthesia formulated by Overton[25] and Meyer and Hemmi[26] predicts that the concentration of anesthetic in the membrane–lipid phase is of the order of 0.03—0.06 mol of anesthetic per liter of membrane lipid. It was also observed that at very high anesthetic concentrations, when all the anesthetic sites are saturated, the membrane concentration is of the order of 0.065 mol per kg dry membrane for the anesthetic alcohols[27] and 0.08 mol per kg for chlorpromazine,[24] and that these high membrane concentrations of anesthetics, which correspond to around one anesthetic molecule per five phospholipid molecules in erythrocyte membrane, are invariably associated with the threshold for membrane lysis.[28] The membrane concentrations obtained by Colley *et al.*[28] are not fully in accordance with ours. The partial discrepancy may be due to the difference in methodology, since they[28] and others[24,27] have all measured the radioactivity in the supernatant after centrifugation, without washing the membrane. There are many and serious doubts about the conventional centrifugation method: 1) the drug precipitated together with the membranes or cells consists of the drug bound to the surface by electrostatic and hydrophobic interactions as well as the penetrated drug, and 2) the drug micelles formed at higher concentrations (*e.g.* above 7×10^{-4} M for chlorpromazine[17]) are precipitated by centrifugation, and are thus measured as penetrated or bound drug, so that abnormally high values of drug penetrated are obtained. Luxnat and Galla also suggested that in the centrifugation technique, these micelles are pelleted together with the cells, leading to erroneously high partition coefficients.[29] Therefore, the net amount of drug penetrated should be determined after washing of the membranes or cells to remove the drug bound to the cells or membranes, and micelles.

The interaction of chlorpromazine with the membrane is hydrophobic [24,30] and anesthetics are partly or completely surrounded by hydrophobic components of the membrane.[24,27,31] Our previous studies indicated that the drugs disturb the arrangement of phospholipids and the hydrophobic interactions between lipids and proteins,[17] and that the boundary phospholipids of Mg^{2+}-adenosine triphosphatase in the membrane are measurably influenced by the drugs.[18] Therefore, the drugs penetrated into the membrane probably exist in the non-polar portions of lipid molecules and at the interfaces between lipid and protein molecules.

The activation energies for drug penetration were 1.07 to 6.16 kcal. However, most values ranged from 3.13 to 6.16 kcal, except for perazine and prochlorperazine. The low activation energies for the latter two drugs may be accounted for by the presence of the piperazinyl ring in the side chain of these drugs; the bulky ring presumably perturbs the membrane structure effectively even at the lower temperature.

Conrad and Singer have reported that the amphipaths, including many anesthetics and tranquilizers, are excluded from biological membranes but are readily soluble in artificial bilayer membranes.[32] They interpret these results by assuming an "internal pressure" in biological membranes.[32] Recently, Luxnat *et al.* investigated the binding of chlorpromazine to artificial and erythrocyte bilayer membranes[33]; their findings are not consistent with the

observations of Conrad and Singer,[32] and they claimed that the partition coefficient is dependent on membrane composition. Our results also cast serious doubt on the interpretation of Conrad and Singer that amphipaths are completely excluded from biological membranes, and demonstrate that the amphipaths are partitioned into or are soluble in erythrocyte membrane quite readily.

In conclusion, we consider that our method for measuring drug penetration yields values close to the net partition of a drug into the erythrocyte membrane as compared with the conventional centrifugation technique. It was found that drugs exerting strong hemolytic action can penetrate into the cell membrane more easily than those with lower activity. The drug penetration into erythrocyte membrane at the concentrations tested could be approximately analyzed by means of Freundlich plots.

Acknowledgement We thank the pharmaceutical companies cited in the text for the supply of drugs.

References

1) H. Chaplin, Jr., H. Crawfold, M. Cutbush and P. L. Mollison, *J. Clin. Pathol.*, **5**, 91 (1952).
2) A. R. Freeman and M. A. Spirtes, *Biochem. Pharmacol.*, **11**, 161 (1962).
3) A. R. Freeman and M. A. Spirtes, *Biochem. Pharmacol.*, **12**, 47 (1963).
4) A. R. Freeman and M. A. Spirtes, *Biochem. Pharmacol.*, **12**, 1235 (1963).
5) P. M. Seeman and H. S. Bialy, *Biochem. Pharmacol.*, **12**, 1181 (1963).
6) G. Zografi, D. E. Auslander and P. L. Lytell, *J. Pharm. Sci.*, **53**, 573 (1964).
7) L. L. M. van Deenen and R. A. Demel, *Biochim. Biophys. Acta*, **94**, 314 (1965).
8) P. Seeman and J. Weinstein, *Biochem. Pharmacol.*, **15**, 1737 (1966).
9) S. Roth and P. Seeman, *Biochim. Biophys. Acta*, **255**, 190 (1972).
10) J. van Steveninck, W. K. Gjösûnt and H. L. Booij, *Biochem. Pharmacol.*, **16**, 837 (1967).
11) S. Roth and P. Seeman, *Nature* (London), *New Biol.*, **231**, 284 (1971).
12) P. Seeman, *Biochem. Pharmacol.*, **15**, 1753 (1966).
13) W. O. Kwant and J. van Steveninck, *Biochem. Pharmacol.*, **17**, 2215 (1968).
14) I. P. Lee, *J. Pharmacol. Exp. Ther.*, **196**, 525 (1976).
15) T. Ogiso, S. Imai, R. Hozumi, M. Kurobe and Y. Kato, *Chem. Pharm. Bull.*, **24**, 479 (1976).
16) T. L. Miller and D. R. Buhler, *Biochim. Biophys. Acta*, **352**, 86 (1974).
17) T. Ogiso, M. Kurobe, H. Masuda and Y. Kato, *Chem. Pharm. Bull.*, **25**, 1078 (1977).
18) T. Ogiso, M. Iwaki and K. Mori, *Biochim. Biophys. Acta*, **649**, 325 (1981).
19) T. Ogiso, M. Iwaki and M. Kuranari, *Chem. Pharm. Bull.*, **31**, 4508 (1983).
20) J. T. Dodge, C. Mitchell and D. J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).
21) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
22) A. Otsuka and H. Sezaki, "Martin Physical Pharmacy, Physical Chemical Principles in the Pharmaceutical Sciences," No. 2, ed. by A. Martin, J. Swarbrick and A. Cammarata, Hirokawa, Tokyo, 1985, pp. 476—478.
23) J. R. Trudell, *Anesthesiology*, **46**, 5 (1977).
24) W. O. Kwant and P. Seeman, *Biochim. Biophys. Acta*, **183**, 530 (1969).
25) E. Overton, *Physiol. Rev.*, **10**, 171 (1930).
26) K. H. Meyer and H. Hemmi, *Biochem. Z.*, **277**, 39 (1935).
27) P. Seeman, S. Roth and H. Schneider, *Biochim. Biophys. Acta*, **225**, 171 (1971).
28) C. M. Colley, S. M. Metcalfe, B. Turner, A. S. V. Burgen and J. C. Metcalfe, *Biochim. Biophys. Acta*, **233**, 720 (1971).
29) M. Luxnat and H. Galla, *Biochim. Biophys. Acta*, **856**, 274 (1986).
30) H. Balzer, M. Makinose and W. Hasselbach, *Arch. Exp. Pathol. Pharmakol.*, **260**, 444 (1968).
31) S. Roth and P. Seeman, *Biochim. Biophys. Acta*, **255**, 207 (1972).
32) M. J. Conrad and S. J. Singer, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 5202 (1979).
33) M. Luxnat, H. Müller and H. Galla, *Biochem. J.*, **224**, 1023 (1984).