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Depression of Phase-transition Temperature in a Model Cell Membrane by Local Anesthetics

Issaku Ueda, M.D., Ph.D., Chikara Tashiro, M.D.,† Kasumi Arakawa, M.D.‡*

Disordering, fluidizing and dilating effects of anesthetics upon cell membranes are well recognized. The fluidization can be precisely measured with phospholipid membranes. When phospholipids are dispersed in water, they form globules of bilayer structure. These model membranes undergo transition between crystalline (ordered and less fluid) and liquid crystalline (less ordered and fluid) phases according to the temperature, the degree of packing of each molecule, and the chemical environment. The phase transition occurs in a cooperative fashion and the turbidity of the dispersion changes abruptly, clear in liquid crystalline phase and turbid in crystalline phase. The present study was undertaken to quantitate the fluidizing effects of local anesthetics on dipalmitoyl lecithin (DPL) bilayer dispersion by measuring the turbidity change. Tetracaine, bupivacaine, lidocaine, and procaine were studied. They all depressed the phase-transition temperature. The binding of the drugs to the model membrane followed unsaturable kinetics, and the *p*H titration curve showed that only uncharged molecules were active. The freezing point depression was analyzed according to the Van't Hoff model. From this model, the partition coefficients of the uncharged molecules between DPL and water were estimated: lidocaine 76, procaine 159, bupivacaine 812, and tetracaine 1,405. The concentration of local anesthetics in the DPL phase needed to decrease the phase-transition temperature 1 degree C showed a constant value of 0.132 M. The concentration of local anesthetics in the DPL phase is a function of *p*H, partition coefficient, and volume ratio between the DPL and aqueous phases. The normalized values of the fluidizing action of these drugs at physiologic conditions correlated well with their nerve-blocking potencies. The present results indicate that the uncharged molecules fluidize the lecithin membrane by unsaturable nonspecific binding. The possible effect of the charged molecules upon the fluidity of natural membranes remains to be established. (Key words: Theories of anesthesia, local anesthetics; Membrane models; Membrane, lipid bilayers; Liposomes; Phase transition.)

THE FLUIDIZING and disordering actions of local anesthetics upon phospholipid membranes have been investigated with electron spin resonance spectroscopy. Hubbell *et al.*,[1] Trudell *et al.*,[2] Butler *et al.*,[3,4] and Hsia and Boggs[5] found with various nitroxide-labeled lipids as spin probes in nerve axons, erythrocytes, or phospholipid membranes that local anesthetics decreased the spectral anisotropy (orderliness) of the spin label, indicating disorder among the phospholipid matrices. Ueda *et al.*[6] demonstrated that general anesthetics promoted a gel-to-liquid crystalline transition of phospholipid monolayers and increased membrane fluidity.

The transition between gel and liquid crystalline phases in phospholipid globules (liposomes) dispersed in the aqueous medium is accompanied by a sudden change in turbidity, which can be monitored by measurement of optical density or light scattering. Hill[7] reported a depression of the phase-transition temperature of phospholipid liposomes by general anesthetics, measured by the change of optical density. The present communication reports the effects of local anesthetics upon thermotropic phase transition of dipalmitoyl lecithin bilayer liposome, measured by light scattering.

Method

Synthetic dipalmitoyl lecithin (1.2-dihexadecyl-*sn*-glycero-3-phosphorylcholine, DPL) was obtained from Calbiochem. Its purity was checked by thin-layer chromatography using chloroform–methanol–water 65:25:4 (v/v/v) as a solvent and found to show a single spot. The purity was further confirmed by proton nuclear magnetic resonance spectroscopy of the material dissolved in deuterated chloroform. All other chemicals were reagent grade.

Water was purified by distillation, followed by ion-exchange, treatment with activated charcoal, and ultrafiltration in a Millipore water-purifying system. Absence of surface active impurities was ascertained by measuring the dynamic surface tension of the water. When the surface tension was decreased more than 0.5 dynes/cm by the compression of the surface area, contamination was suspected.

DPL was dispersed in water at a concentration of 0.5 , 1.0, 1.5, 2.5 or 5.0 mM at 45 C (above the phase-transition temperature) with ultrasonic irradiation. The *p*H was adjusted with hydrochloric acid or sodium hydroxide. An Orion 701 *p*H meter and a glass electrode were used for *p*H determinations.

A 3.0-ml volume of the sonicated liposome dispersion was placed in a 1.0-cm light-path fluorometry cuvette, to which 0.1 ml of water or local anesthetic was added, and placed in a thermostatted cuvette compartment of a Hitachi Perkin-Elmer double-beam spectrofluorometer. The cuvette temperature was monitored with a thermistor probe inserted into the cuvette and a Digitec thermometer. Temperature was controlled by circulating water from a Haake water bath. Turbidity was measured by

* Professor.

† Research Fellow. Present address: Department of Anesthesia, Osaka University Medical School, Fukushima-Ku, Osaka, Japan.

‡ Associate Professor and Acting Chairman.

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Address reprint requests to Dr. Ueda.

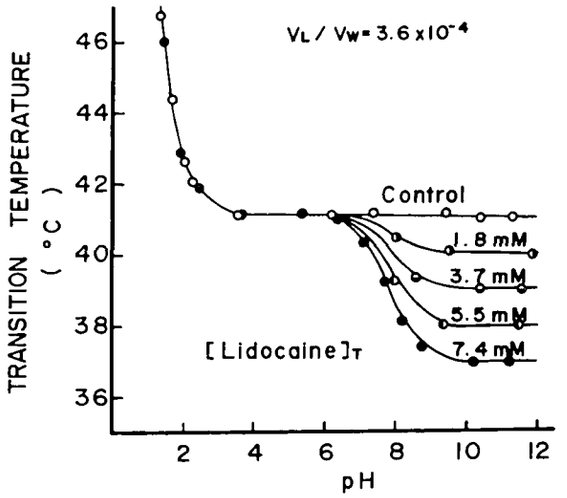

FIG. 1. The effects of lidocaine upon the phase-transition temperature at a concentration of 0.5 mM DPL (volume ratio of 3.6×10^{-4}). The lidocaine concentrations are indicated. Curves are drawn according to equation 17. The perfect fits to the experimental data points demonstrate that only uncharged molecules are effective.

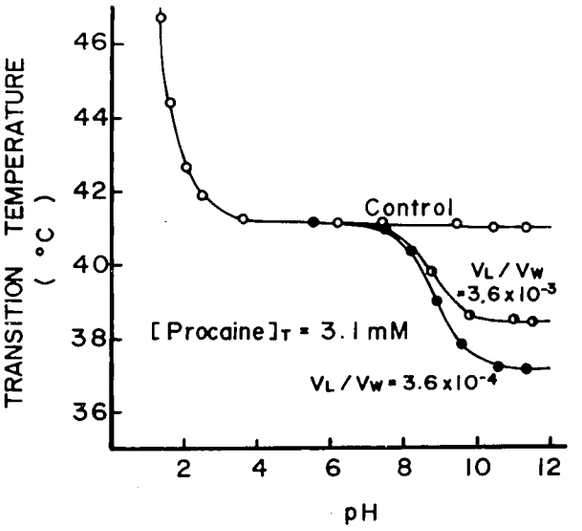

FIG. 2. The effects of phospholipid concentrations upon the procaine action on the phase-transition temperature. The procaine concentration was 3.1 mM. The DPL concentrations are expressed in a volume ratio against water volume (V_L/V_W) and are indicated in the figure. The curves are drawn according to equation 17 and they fit the experimental points perfectly. The depressant action was stronger when the V_L/V_W ratio was smaller.

90-degree light scattering at a wavelength of 360 nm and recorded on a strip-chart recorder. Temperatures were decreased at a rate of 1 degree C/3 min and the point where a sudden increase of the intensity of scattered light occurred was taken as the phase-transition temperature.

The extent of charged and uncharged molecules contributing to the depression of the phase-transition temperature was estimated from the pH titration curve of the drug activity. Because our results established that only uncharged molecules were active in depressing the phase-transition temperature, further data were obtained at pH values where more than 99 per cent of the local anesthetics were in uncharged forms ($p\text{H} - p\text{K}_a > 2$).

The effects of local anesthetics on the phase-transition temperature were analyzed according to the model described by Van't Hoff, which deals with depression of the freezing point of a solvent by the presence of a solute (see Appendix). The data obtained were plotted between volume ratio of [DPL]/[water] and the ratio of [total concentration of the local anesthetics]/[depression of the phase-transition temperature]. The volume of DPL was calculated from the density of DPL (1.0305). From the slope of this plotting, the magnitudes of depression of the phase-transition temperature by the unit concentrations of the local anesthetics were calculated. From the intercept, the partition coefficients of the local anesthetics between the phospholipid phase and the aqueous phase were obtained.

Results

All local anesthetics lowered the phase-transition temperature when the pH was near or above the $p\text{K}_a$ of the compound (fig. 1). These pH titration curves indicate that only uncharged molecules are effective. A theoretical curve that assumes only uncharged molecules are effective (equation 17, Appendix) was constructed. The experimental data showed a perfect fit to the theoretical curve. The magnitude of depression of the phase-transition temperature was directly proportional to the amount of uncharged anesthetic molecules present. This study was performed with constant DPL concentrations.

Because the concentrations of local anesthetics partitioned into the DPL phase bear an inverse relationship to the relative volume of DPL in the medium (equation 10, Appendix), the effect of DPL concentration upon the depression of the phase-transition temperature were investigated. Again, the experimental data points fit perfectly to the theoretical curves (fig. 2), showing that decrease of the phospholipid concentration increased drug action.

When local anesthetics were absent, the phase-transition temperature (T_c) was unaffected by changes of DPL concentrations in a range between 0.5 and 5.0 mM. The T_c–pH curve without local anesthetics (figs. 1 and 2) showed a straight line when the pH was above 4.0. The phase-transition temperature was about 41.3 C, and it decreased only slightly as pH was increased. The following relationship was found between the phase-transition temperature (T_c) and pH.

$$T_c = 41.29 - 0.026 \times p\text{H}(x) \qquad (1)$$

Below pH 3.5, the phase-transition temperatures increased sharply.

When more than 99 per cent of the local anesthetics were in the uncharged forms, the increase of DPL concentrations from 0.5 to 5.0 mM increased the concentrations of procaine necessary to depress the phase-transition temperature one degree C from 0.797 to 1.177 mM. The data for the other anesthetics were: lidocaine, 1.808 to 2.232 mM; bupivacaine, 0.213 to 0.642 mM; tetracaine, 0.148 to 0.591 mM. Table 1 lists the concentrations of the local anesthetics needed to depress the phase-transition temperature 1 degree C at various volume ratios of DPL to water.

The dose–response relationships between the concentrations of the uncharged species of lidocaine, procaine, bupivacaine, and tetracaine and the decrease of the phase-transition temperature were found to be linear, showing unsaturable binding kinetics and nonspecific association of the anesthetic molecules with DPL (fig. 3). Figure 3 tends to give an impression that the effects do not correlate well with the clinical potencies. However, it must be emphasized that the plotted concentrations of anesthetics are uncharged species, and when they are normalized to physiologic pH and total concentrations of the drugs, good correlation with the clinical potencies is obtained.

When [total anesthetic concentration]/[depression of phase-transition temperature] is plotted against [volume of DPL]/[volume of water], a straight line should be obtained; the partition coefficients between DPL and water can be calculated from its intercept, and the magnitude of depression of the phase-transition temperature by unit concentrations of anesthetics can be obtained from its slope (Appendix). The plot produced parallel lines among the four anesthetics (fig. 4). These parallel lines indicate that the magnitude of depression of the phase-transition temperature shows a constant value among these drugs. The value is calculated to be 7.6 degrees C per 1 M, or conversely, 0.132 M per 1 degree C. The partition coefficients of the uncharged molecules of these drugs between the DPL phase and water were: lidocaine 76, procaine 159, bupivacaine 182, and tetracaine 1,405.

TABLE 1. Alteration of Local Anesthetic Action by Changes in Ratio between DPL and Water Volumes (or DPL Concentrations)

	Lecithin 0.5 mM V_L/V_W 0.356 $\times 10^{-3}$	Lecithin 1.0 mM V_L/V_W 0.712 $\times 10^{-3}$	Lecithin 1.5 mM V_L/V_W 1.069 $\times 10^{-3}$	Lecithin 2.5 mM V_L/V_W 0.781 $\times 10^{-3}$	Lecithin 5.0 mM V_L/V_W 3.562 $\times 10^{-3}$
Procaine (mmol/l)	0.797	0.827	0.874	0.956	1.177
Lidocaine (mmol/l)	1.808	1.845	1.892	1.942	2.232
Bupivacaine (mmol/l)	0.213			0.385	0.642
Tetracaine (mmol/l)	0.148			0.325	0.591

* The concentrations of local anesthetics that depressed the phase-transition temperature one degree C at various DPL/water volume ratios are presented. The data were obtained at pH values where more than 99 per cent of the local anesthetics were in uncharged forms.

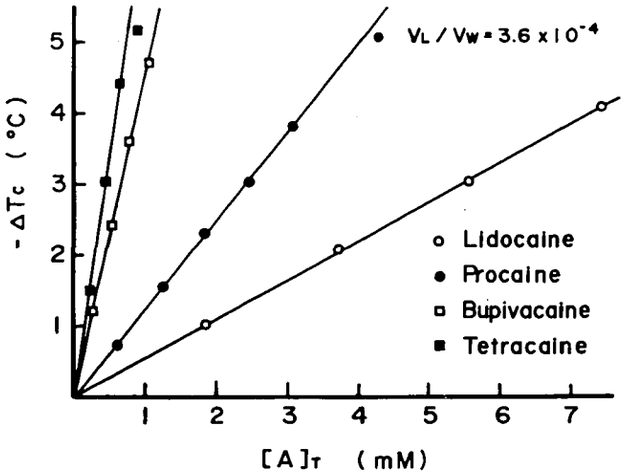

FIG. 3. The effects of four local anesthetics upon the depression of the phase-transition temperature. The data were collected at a pH value two units above the pK_a of each local anesthetic. The phospholipid concentration was constant at 0.5 mM. This dose–response relationship indicates unsaturable binding of uncharged molecules to the DPL phase. Apparent deviations from the clinical potencies are due to the difference of pH. Normalized values correlate well with the blocking potencies (see Discussion).

Discussion

It appears to be a common belief among anesthesiologists that local anesthetics penetrate the cell membrane in uncharged form and bind to the membrane in charged form from the inside of the membrane. This concept was first proposed by Ritchie and Greengard.[8]

Direct evidence that charged molecules of dissociable local anesthetics are effective in conduction block was demonstrated by Narahashi *et al.*[9] with internally perfused squid giant axons. Their results showed that only charged molecules blocked nerve conduction when given inside of the axon, and favor the view that site-specific receptor-oriented binding is responsible for conduction block. Nevertheless, the concept that only the charged species are active is difficult to reconcile with the fact that totally uncharged molecules like alcohols and benzocaine are good local anesthetics, as Ritchie[10] has pointed out. Presumably, both charged and uncharged molecules contribute to the mechanism of local anesthesia.

The present results demonstrate that only uncharged species are active in DPL model membranes for depression of the phase-transition temperature. The binding of the local anesthetics showed unsaturable kinetics and obviously was not receptor-oriented. The uncharged molecules partition into the DPL phase, presumably by hydrophobic interactions. The partition coefficients of the four drugs studied were estimated.

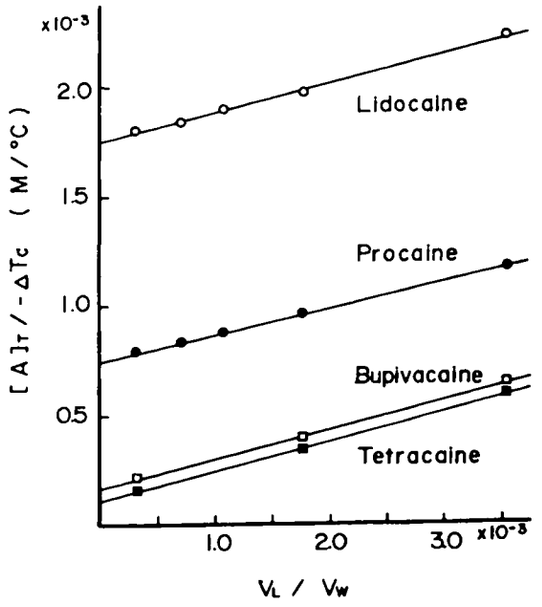

FIG. 4. Plotting of equation 16. From the slope and the intercept, k and partition coefficients are obtained. Because all four lines are parallel the slope has a constant value of 7.6 degrees C per M or 0.132 M per 1 degree C. The values for the partition coefficients of the anesthetics are listed in table 2.

The order of the values of the partition coefficients was tetracaine > bupivacaine > procaine > lidocaine. The dose–response relationships depicted in figure 3 show the same order of the potencies of these drugs in depressing the phase-transition temperature. These orders do not appear to correlate with nerve-blocking potencies. However, the effects of these drugs upon phase-transition temperature are a function of *p*H, which determines the ratio between the charged and uncharged species, partition coefficient, and the volume ratio between DPL and water. The local anesthetic actions are meaningfully analyzed only when these three variables are properly considered.

TABLE 2. k Values and Partition Coefficients Calculated from the Slope and Intercept of Equation 16 (Fig. 4)

					$-\Delta T_c/[A]_T$ (Relative Potency)			
					$V_L/V_W = 1/\infty$		$V_L/V_W = 1/50$	
	pK_a	$\frac{1/k\ [A]_L}{-\Delta T_c}$ M/°C	$\frac{k\ -\Delta T_c}{[A]_L}$ °C/M	$\frac{P\ [A]_L}{[A]_W}$	*p*H 7.2 °C/mM	*p*H 7.4 °C/mM	*p*H 7.2 °C/mM	*p*H 7.4 °C/mM
Procaine	8.90	0.120	8.3	159	0.03 (1.0)	0.04 (1.0)	0.03 (1.0)	0.04 (1.0)
Lidocaine	7.87	0.133	7.5	76	0.10 (3.9)	0.14 (3.5)	0.08 (3.2)	0.11 (2.7)
Bupivacaine	8.05	0.134	7.4	812	0.73 (23.6)	1.07 (21.9)	0.25 (8.5)	0.28 (6.3)
Tetracaine	8.50	0.139	7.2	1,405	0.48 (16.7)	0.75 (16.6)	0.21 (7.6)	0.25 (5.9)

* The values for k average 7.6 degrees C/M or, conversely, 0.132 M/1 degree C. The right side of the table lists the drug activities corrected for *p*H 7.2 and *p*H 7.4 in two DPL concentrations. Figures in parentheses are the relative potencies, taking procaine as a reference.

A comparison of the present data with those obtained in studies of conduction blocks of excised nerves[11] was attempted by taking the volume ratio between DPL and water (V_L/V_W) to be $1/\infty$, at *p*H 7.4. When the local anesthetics were present at an overall concentration of 1 mM in the whole system (DPL and water phases together), the degrees of depression of the phase-transition temperature were calculated to be: procaine, 0.04 degrees C; lidocaine, 0.14 degrees C; bupivacaine, 1.07 degrees C; tetracaine, 0.75 degrees C. When these values are converted to relative potencies, taking procaine as a reference, the following values are obtained: procaine 1.0, lidocaine 3.5, bupivacaine 21.9, and tetracaine 16.6. At another arbitrary value of $V_L/V_W = 1/50$ and at *p*H 7.4, the relative potencies are: procaine 1.0, lidocaine 2.7, bupivacaine 6.3, and tetracaine 5.9 (table 2). These values correlate reasonably well with the relative potencies of these drugs to induce conduction blocks. Lunt *et al.*[11] listed relative potencies of procaine, lidocaine, and bupivacaine in isolated frog sciatic nerve to be 1:4:16, and those in epidural anesthesia to be 1:2:8.

The macromolecules of cell membrane proteins are solvated into the phospholipid matrices. The liquidity of the phospholipid domain is expected to influence the protein conformation and the function of the cell membrane. The good correlation found in the present data between the effect of these dissociable local anesthetics upon the depression of the phase-transition temperature and clinical potency suggests that the increase of the fluidity by the uncharged molecules constitutes a certain part of the mechanism of local anesthesia.

Shauf and Agin[12] showed that the combined effects of a dissociable local anesthetic, procaine, and an undissociable local anesthetic, benzyl alcohol, upon lobster giant axon were additive. They postulated that the mechanisms of the actions of these two drugs should be similar, and the uncharged molecules of procaine may be active in blocking conduction. A similar additive inhibitory effect of dissociable and undissociable local anesthetics on ATP-induced luminescence of firefly lantern extract was reported by Kamaya *et al.*[13] A thermodynamic analysis of the interaction of dissociable local anesthetics with the firefly luminescent system revealed both specific and nonspecific association of these drugs with the enzyme, indicating that both charged and uncharged molecules were effective in suppressing the light intensity.[14]

Pressure reversal of anesthesia is now established as an important feature of the actions of general anesthetics. It represents hydrophobic interaction of

uncharged molecules with protein or membrane structure, resulting in the conformational changes and dilatation. Although recent reports on the pressure antagonism of local anesthetic actions are conflicting, with both positive[15] and negative[16] results, Johnson *et al.*[17] originally reported that the inhibition of bacterial luminescence induced by procaine was reversed only partially by the hydrostatic pressure. It is tempting to speculate that both charged and uncharged molecules inhibit the light intensity, and that only the effects of the uncharged molecules are reversible by the pressure. The interaction of charged molecules may not induce a gross conformational change of the light-emitting enzyme.

The generalization that the cell membrane is penetrated by uncharged forms and conduction block is achieved only by charged molecules is an apparent oversimplification. Presumably, both charged and uncharged molecules may participate in local anesthesia by different mechanisms. The mode of action of uncharged molecules is to induce disorder among the lipid part of the cell membrane, fluidizing and expanding it by nonspecific binding. Charged molecules may inhibit nerve conduction by receptor-oriented specific binding. The extent of the contribution of each mechanism to conduction block is an open question.

Sterling-Winthrop Research Institute provided tetracaine, bupivacaine and procaine, and Astra Pharmaceutical Products, Inc., provided lidocaine for use in this study.

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APPENDIX

In acidic medium, uncharged local anesthetics, A, are protonated to form charged species, AH^+, which dissociate into A and H^+ according to the dissociation constant K_a.

$$AH^+ \rightleftharpoons A + H^+ \quad (2)$$

$$K_a = [A][H^+]/[AH^+] \quad (3)$$

For convenience we write $[A]/[AH^+] \equiv a$, then

$$a \equiv [A]/[AH^+] = K_a/H^+ = 10^{pH-pH_a} \quad (4)$$

According to the present result, only [A] partitions into DPL with a coefficient P. Using subscripts W and L to denote aqueous and DPL phases, respectively,

$$[A]_L/[A]_W = P \quad (5)$$

From equation 4,

$$[A]_W = a[AH^+]_W \quad (6)$$

At equilibrium, from equations 5 and 6,

$$[A]_L = a \cdot P[AH^+]_W \quad (7)$$

When a local anesthetic is added to a final concentration of $[A]_T$ in a DPL liposome system that has a volume of $V_T = V_W + V_L$, where V_W and V_L are the volumes of water and DPL, respectively, the conservation equation can be written as,

$$V_T[A]_T = (V_W + V_L)[A]_T$$
$$= V_W[AH^+]_W + V_W[A]_W + V_L[A]_L \quad (8)$$

$$= [AH^+]_W(V_W + a \cdot V_W + a \cdot P \cdot V_L)$$

$$[AH^+]_W = \{(V_W + V_L)[A]_T\}/\{V_W + a \cdot V_W + a \cdot P \cdot V_L)\} \quad (9)$$

From equations 7 and 9,

$$[A]_L = \frac{a \cdot P(V_W + V_L)}{V_W + a(V_W + PV_L)} [A]_T \quad (10)$$

According to the Van't Hoff model, the depression of the phase transition temperature ($-\Delta T_c$) is proportional to the molal concentration of solute $[A]_L'$ in the solvent DPL.

$$-\Delta T_c = k'[A]_L' \quad (11)$$

The density of DPL is close to unity and show a value of 1.0305. When the volume of the solute is much smaller than the solvent, equation 11 becomes

$$-\Delta T_c = k[A]_L \quad (12)$$

From equations 10 and 12, we obtain

$$-\Delta T_c = \frac{a \cdot P(1 + V_L/V_W)}{1 + a(1 + PV_L/V_W)} k[A]_T \quad (13)$$

When $p\mathrm{H} - p\mathrm{K_a} \gg 2$, $[AH^+]_W$ becomes negligible in equation 8 and equation 13 reduces to

$$-\Delta T_c = \frac{P(1 + V_L/V_W)}{1 + P \cdot V_L/V_W} k[A]_T \quad (14)$$

By rearranging,

$$\frac{[A]_T}{-\Delta T_c} = \frac{1}{k}\left(\frac{1}{P} + \frac{V_L}{V_W}\right) \Big/ \left(1 + \frac{V_L}{V_W}\right) \quad (15)$$

In the present experiment, $V_L/V_W \ll 1$. For example, the highest concentration of DPL was 5 mM and $V_L/V_W = 3.562 \times 10^{-3}$. Therefore,

$$\frac{[A]_T}{-\Delta T_c} = \frac{1}{k}\left(\frac{1}{P} + \frac{V_L}{V_W}\right) \quad (16)$$

By plotting V_L/V_W against $[A]_T/-\Delta T_c$, a straight line should be obtained, and the values for k and P are obtained from the slope and the intercept of the straight line.

From equations 1 and 14, the phase transition temperature, T_c, at a pH value of x in the presence of local anesthetics can be computed by,

$$T_c = 41.29 - 0.026x - k\frac{P(1 + V_L/V_W)10^{x-p\mathrm{K_a}}}{1 + (1 + P \cdot V_L/V_W)10^{x-p\mathrm{K_a}}} [A]_T \quad (17)$$

Erratum

An error appeared in the article "Urinary Excretion of Morphine during and after Valvular and Coronary-artery Surgery" in the March 1977 issue. The third and second lines from the bottom of page 167 in the left hand column now read ". . . while those of patients with mitral-valve and aortic-valve disease were extubated 14.6 ± 3.1 and 20.9 ± 6.3 hours postoperatively." They should read ". . . while those of patients with aortic-valve and mitral-valve disease were extubated 14.6 ± 3.1 and 20.9 ± 6.3 hours postoperatively."