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**LA THÈSE A ÉTÉ
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Yvan Boulanger

Study of Local Anesthetic-Membrane
Interactions by Deuterium and Phosphorus-31
Nuclear Magnetic Resonance

Thesis presented to the
School of Graduate Studies
University of Ottawa
In partial fulfilment of the
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1980



Yvan Boulanger, Ottawa, Canada, 1980.

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ABBREVIATIONS

BPC	Borate-phosphate-citrate buffer
DMPC	1,2-dimyristoyl- <u>sn</u> -glycero-3-phosphocholine
DPFC	1,2-dipalmitoyl- <u>sn</u> -glycero-3-phosphocholine
DPFC-2'-d ₂	1-palmitoyl-2-palmitoyl-2',2'-d ₂ - <u>sn</u> -glycero-3-phosphocholine
DPFC-6'-d ₂	1-palmitoyl-2-palmitoyl-6',6'-d ₂ - <u>sn</u> -glycero-3-phosphocholine
DPFC-12'-d ₂	1-palmitoyl-2-palmitoyl-12',12'-d ₂ - <u>sn</u> -glycero-3-phosphocholine
DPFC-16'-d ₃	1-palmitoyl-2-palmitoyl-16',16',16'-d ₃ - <u>sn</u> -glycero-3-phosphocholine
DPFC-d ₆₂	1,2-dipalmitoyl-d ₆₂ - <u>sn</u> -glycero-3-phosphocholine
DPSS	1,2-dipalmitoyl- <u>sn</u> -glycero-3-phosphoserine
ESR	Electron spin resonance
FID	Free induction decay
FT	Fourier transform
GLC	Gas-liquid chromatography
IR	Infrared spectroscopy
NMR	Nuclear magnetic resonance

PC-d ₄	<u>sn</u> -3-phosphatidylcholine-3,3,4,4-d ₄
PC-d ₉	N,N,N-trimethyl-d ₉ - <u>sn</u> -glycero-3-phospho- choline or <u>sn</u> -3-phosphatidylcholine-d ₉
PC	<u>sn</u> -3-phosphatidylcholine (natural source)
PE	<u>sn</u> -3-phosphatidylethanolamine (natural source)
PE-d ₄	<u>sn</u> -3-phosphatidylethanolamine-2,2,3,3-d ₄
PI	<u>sn</u> -3-phosphatidylinositol (natural source)
PRC	Procaine hydrochloride
PRC-d ₄	Procaine hydrochloride-d ₄ or 2-diethyl-2,2, 2',2'-d ₄ -aminoethyl-4-aminobenzoate hydro- chloride
PRC-d ₂	Procaine hydrochloride-d ₂ or 2-diethylami- noethyl-4-aminobenzoate-3,5-d ₂ hydrochlo- ride
PS	<u>sn</u> -3-phosphatidylserine (natural source)
RMN	Résonance magnétique nucléaire
TTC	Tetracaine hydrochloride
TTC-d ₂	Tetracaine hydrochloride-d ₂ or 2-dimethyl- aminoethyl-4-butylaminobenzoate-3,5-d ₂ hydrochloride
TTC-d ₃	Tetracaine hydrochloride-d ₃ or 2-dimethyl- aminoethyl-4-butyl-4,4,4-d ₃ -aminobénzoate hydrochloride

TTC-d ₆	Tetracaine hydrochloride-d ₆ or 2-dimethyl-d ₆ -aminoethyl-4-butylaminobenzoate hydrochloride
UV-VIS	Ultraviolet and visible spectroscopy

FIGURES

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(32 mM lipid in 1.0 ml buffer). The spectra were acquired with the quadrupole echo technique ($\tau_1 = 60 \mu\text{s}$, $\tau_2 = 50 \mu\text{s}$), 2048 data points, a 500 kHz spectral width, a 4.8 μs (PC-12'-d₂) or 8 μs (PC-d₄) 90° pulse, 50,000 (PC-12'-d₂) or 15,000 (PC-d₄) scans and a 0.08 s (PC-12'-d₂) or 0.2 s (PC-d₄) recycle time.

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acquired with the quadrupole echo technique ($\tau_1 = 60 \mu\text{s}$, $\tau_2 = 50 \mu\text{s}$) with 2048 data points a 500 kHz spectral width, a 0.2 s recycle time 12,000 scans and a $5 \mu\text{s}$ 90° pulse length.

- Fig. 34 Graph of the ratios of quadrupole splittings (D/D_0) of deuterated PC-PS 1:1 dispersions as a function of the amount of TTC bound (each unit corresponds to 0.05 mole TTC/mole PC).
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TABLES

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PREFACE

Depuis la découverte de l'effet anesthésique de la cocaïne, vers la fin du siècle dernier, le domaine de l'anesthésie s'est développé considérablement tant par l'addition de nouvelles substances que par l'étude de leur effet sur les tissus vivants ou reconstitués. Il existe plusieurs classes d'anesthésiques locaux et généraux et leur usage médical est bien connu. Cependant, à l'exception du fait que ces composés agissent au niveau de la membrane nerveuse, bien peu est connu concernant leur mécanisme d'action. Des théories mettant en cause soit les lipides soit les protéines ont été proposées mais, jusqu'à maintenant, aucune n'a été définitivement établie.

L'interaction de la procaine et de la tétracaine, les deux anesthésiques locaux choisis pour mes études, avec les membranes biologiques ou modèles a été investiguée par différentes techniques qui ont démontré que ces molécules ont un effet sur la bicouche lipidique, cet effet étant plus marqué pour la tétracaine que pour la procaine. Il a été proposé que la forme chargée et/ou la forme non-chargée de l'anesthésique provoque(nt) la perte de sensibilité. De plus, on ignore à peu près

complètement où sera située la molécule d'anesthésique dans la membrane lipidique. Cette étude a pour objectif de clarifier quelques-uns de ces points ambigus.

PREFACE

Since the discovery of the anesthetic effect of cocaine at the end of the last century, the field of anesthesia has considerably developed by the addition of numerous new molecules and study of their action on living systems. Many classes of local and general anesthetics presently exist and are used for clinical purposes. However, little is known about their mechanism of action except that they act at the level of the nerve membrane. Both lipid and protein-based mechanisms are in vogue but, until now, none has been definitely established.

The interaction of procaine and tetracaine, two local anesthetics chosen for this study, with biological or model membranes has been studied by several techniques, which have shown that these molecules interact with the membrane phospholipid bilayer, tetracaine producing a stronger effect than procaine. The charged and/or the uncharged form(s) has (have) been alternately proposed to be responsible for anesthesia. The intercalation of the local anesthetic in the lipid membrane is generally recognized but almost nothing is known about the location of each form. It is the purpose of this work to clarify some of these questions.

RESUME

Des membranes modèles, constituées de dispersions multilamellaires de phospholipides, ont été utilisées pour l'étude de ce que l'on considère comme au moins une étape importante du mécanisme d'anesthésie i.e. l'interaction de l'anesthésique avec la partie lipidique de la membrane.

Je décris ici la synthèse d'anesthésiques locaux, procaine et tétracaine, spécifiquement deutérés ainsi que de phosphatidylcholines spécifiquement deutérées. Après avoir caractérisé le système en déterminant les coefficients de partition, les valeurs de pK_a , les solubilités, etc, l'interaction des anesthésiques marqués avec des membranes modèles de phosphatidylcholine (PC) ou de phosphatidylcholine-phosphatidylsérine (PC-PS) et l'interaction des anesthésiques non-marqués avec des phosphatidylcholines spécifiquement deutérées, ont été étudiées par résonance magnétique nucléaire du deutérium (RMN). Les anesthésiques locaux ont été utilisés dans leurs formes chargée (pH 5.5) et non-chargée (pH 9.5).

Les résultats de RMN peuvent être interprétés par un modèle à trois sites où l'anesthésique serait

(1) libre dans l'eau, (2) faiblement lié et (3) fortement lié à la membrane. Les deux premiers sites sont en échange vite mais l'échange est lent avec le troisième site. Des calculs de pourcentages de l'espèce chargée et non-chargée ont indiqué que les deux formes peuvent être fortement liées à la membrane à bas et à haut pH.

Les résultats de RMN du deutérium pour les anesthésiques locaux suggèrent que les sites de liaison pour les espèces fortement liées chargées et non-chargées sont différents. Les paramètres d'ordre moléculaire de la tétracaine fortement liée indiquent que la molécule se trouve dans un environnement très ordonné dans les deux formes.

Une comparaison des données du RMN du deutérium pour les anesthésiques locaux deutérés en présence de PC et de PC-PS a été utilisée pour évaluer l'importance de l'interaction électrostatique. Dans la plupart des cas, l'addition de PS provoqua une augmentation des effets observés avec PC à bas pH quand l'anesthésique est chargé positivement. Considérant les valeurs beaucoup plus élevées des coefficients de partition quand l'anesthésique est non-chargé, l'interaction hydrophobe demeure supérieure à l'interaction électrostatique, cette dernière jouant un rôle non négligeable.

L'addition d'anesthésique local à des dispersions de PC spécifiquement deutéré a permis la localisation de la molécule dans la bicouche de phospholipides. Dans sa forme chargée, les résultats indiquent que la molécule de tétrocaine est située au niveau du groupe de tête, le groupe diméthylammonium positivement chargé étant dans l'entourage de la partie triméthylammonium de PC. La position du noyau benzénique correspondrait à la partie glycérol du phospholipide et la chaîne N-butyle de tétrocaine s'intercalerait entre les premiers carbones de la chaîne d'acides gras. Dans sa forme non-chargée, le groupe diméthylamine de la tétrocaine semble au niveau du groupe phosphate, l'anneau benzénique dans la région des premiers carbones des chaînes d'acides gras du phospholipide et la chaîne N-butyle au niveau des carbones subséquents. Ces résultats sont aussi en accord avec les données du RMN de ^{31}P pour le groupe phosphate de PC.

ABSTRACT

Model membranes consisting of phospholipid multilamellar dispersions have been used to investigate what is considered at least an important step in the mechanism of anesthesia i.e. the interaction of the anesthetic with the lipidic part of the membrane.

I describe here the synthesis of specifically-deuterated local anesthetics procaine and tetracaine as well as specifically-deuterated phosphatidylcholines. After characterization of the system by determination of partition coefficients, pK_a values, solubility, etc., the interaction of labelled local anesthetics with model membranes of phosphatidylcholine (PC) or phosphatidylcholine-phosphatidylserine (PC-PS) and the interaction of unlabelled local anesthetics with specifically-deuterated phosphatidylcholines, were studied via 2H nuclear magnetic resonance (NMR). The local anesthetics were used in their charged (pH 5.5) and uncharged (pH 9.5) forms.

The NMR results are consistent with a three-site model where the anesthetic is (1) free in water, (2) weakly-bound and (3) strongly-bound to the membrane. A fast exchange exists between the two first sites but exchange is slow with the third site. Calculations of

the percentages of charged and uncharged species indicated that both forms are strongly-bound to the membrane at low (pH 5.5) and high pH (pH 9.5).

The ^2H NMR results for deuterated local anesthetics suggest that the binding sites for the strongly-bound charged and uncharged species are different. Estimates of molecular order parameters for the strongly-bound tetracaine indicate that it is located in a highly ordered environment in both forms.

Comparison of the ^2H NMR data for deuterated local anesthetics interacting with PC and PC-PS was used to monitor the charge interaction. In most cases, effects observed with PC were enhanced upon addition of PS at low pH when the anesthetic is positively charged. The electrostatic interaction plays a role in the anesthetic binding but the hydrophobic interaction remains the main force considering the much stronger binding at high pH when the anesthetic is uncharged.

Addition of local anesthetic to specifically-deuterated PC allowed the location of the former in the phospholipid bilayer. In its charged form, the results suggest that the tetracaine molecule is located at the level of the head group, the positively charged dimethylammonium group being approximately at the level

of the trimethylammonium moiety of PC. The position of the benzenoid ring then corresponds to the glycerol backbone of the phospholipid and the N-butyl chain of tetracaine intercalates between the first carbon segments of the fatty acyl chains. In the uncharged form, the dimethylamino moiety of tetracaine appears to reside at the level of the phosphate group, the benzene ring, in the region of the first carbon segments of the phospholipid fatty acyl chains and the N-butyl chain in the region of the following segments. These results are also supported by ^{31}P NMR data for the phosphate group of PC.

I. INTRODUCTION

A. System

(a) Biological and model membranes

Biological membranes are constituted of lipids and proteins, which are responsible for all the functions played by this universal component of cells: transport, fusion, nerve conduction. These two parts of the membrane are arranged in the form of a layer at the surface of the cell which is usually described by the model of Singer and Nicolson (Fig. 1, ref. 1). In this model, the lipids are placed in a bilayer where the polar groups are outside in contact with water and the apolar chains are inside buried from water. The proteins are randomly intercalated in that bilayer.

Due to the large number of different lipid and protein constituents of biological membranes, it is very difficult to localize specific interactions occurring in natural membranes. In order to simplify the system, model membranes are often used. They consist of lipids which are dispersed in water to form concentric multilamellar dispersions or liposomes of variable sizes (5-50 μ)(Fig. 2, ref. 2-4). Each lamella is a

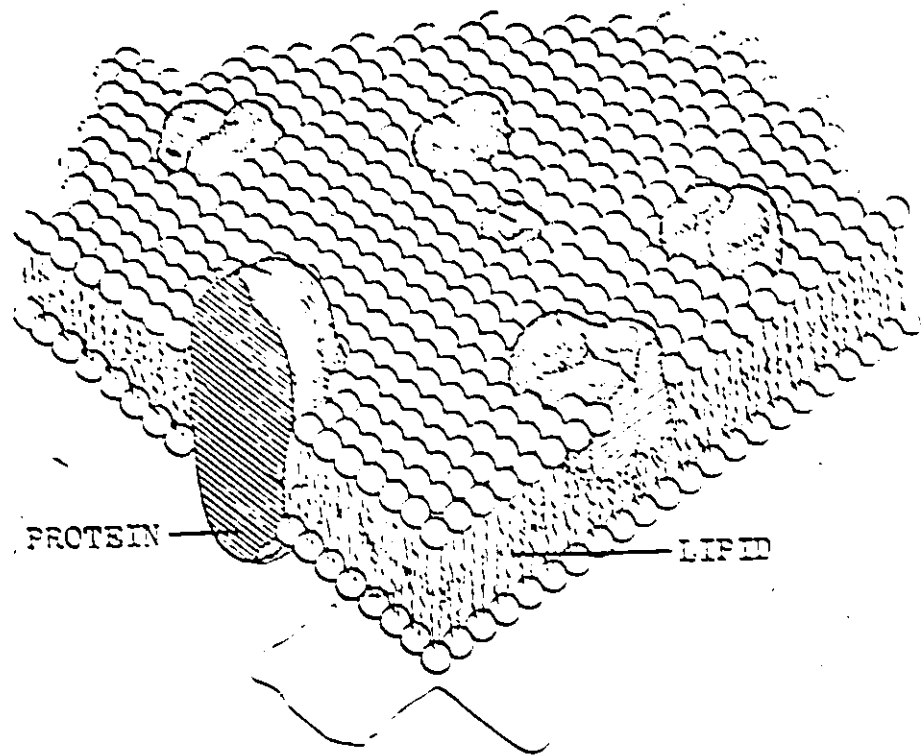


Fig. 1. Fluid-mosaic model of biological membrane proposed by Singer and Nicolson (1). The globular proteins are intercalated in a bilayer array of lipids.

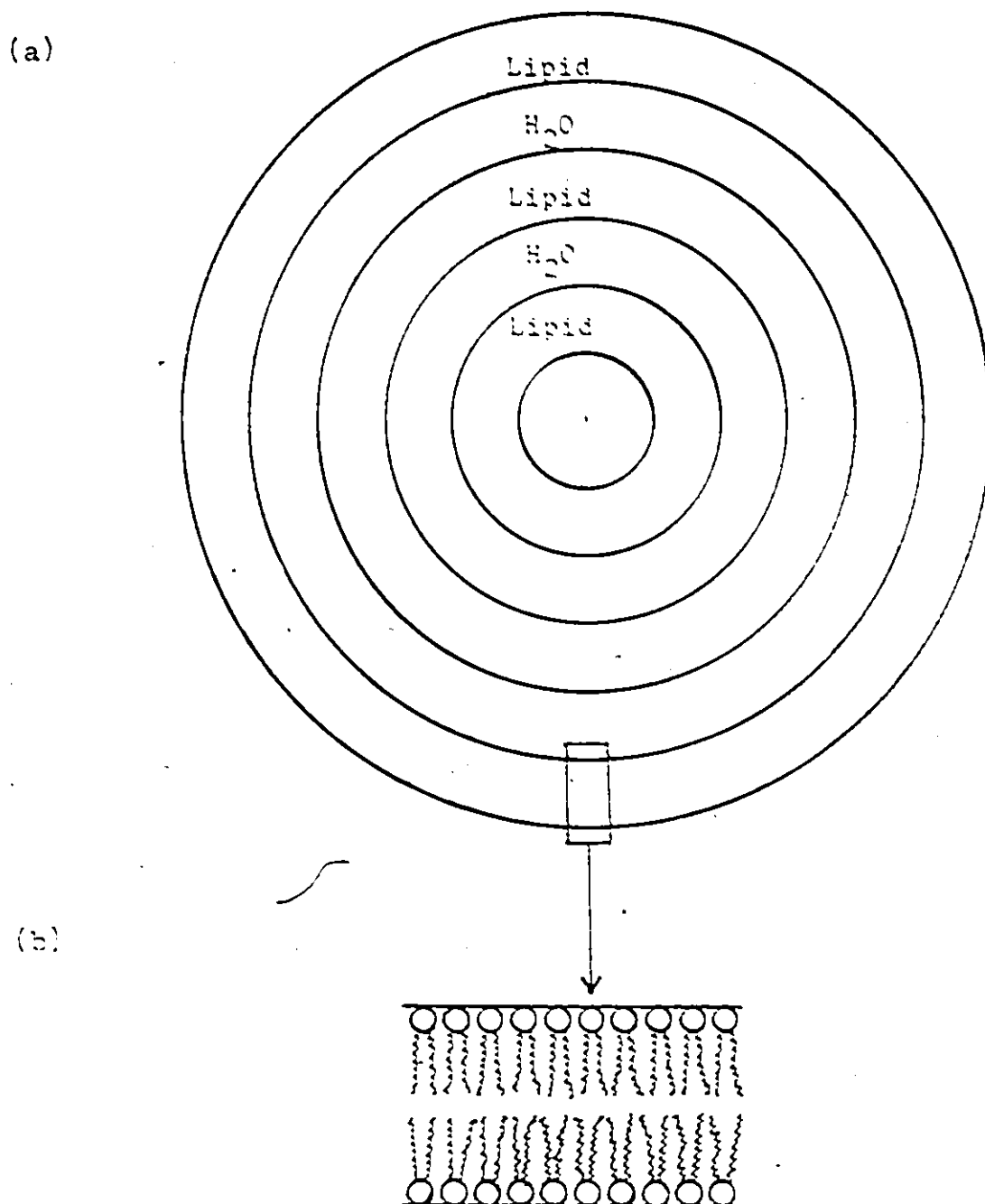


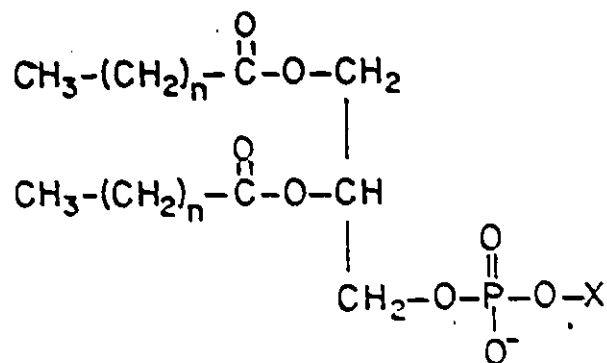
Fig. 2. Structure of a multilamellar lipid dispersion
(a) concentric lamellae of water and lipid bilayers
and (b) bilayer arrangement of the lipids.

bilayer similar to the bilayer formed by the lipids of natural membranes. Bilayers of lipid and water layers are alternating in the multilamellar dispersion. Similarity of the phospholipid arrangement in model and biological membranes has been shown by techniques such as electron microscopy (5,6), X-ray (7,8), neutron diffraction (9), ESR (10,11), NMR (12,13) and others.

Phospholipids, the main class of membrane lipids and the usual component of model membranes, have a structure constituted of a glycerol backbone attached at the sn-1 and ^{sn-2} positions to fatty acid ester groups and in the sn-3 position to different phosphate ester groups (14)(Fig. 3). The phosphate ester group is known as the head group and serves to classify the different types of phospholipids. The phospholipids also differ in the composition of the fatty acyl chains. They can be either ~~natural~~ or synthetic.

The properties of these phospholipids have been summarized earlier (14-16). Dispersions of phospholipids exist in two forms depending on the temperature, gel or liquid crystal. The temperature at which they change from one form to the other is called the transition temperature. Most phospholipid dispersions can also be arranged in a bilayer, hexagonal or isotropic phase

(a)



(b)

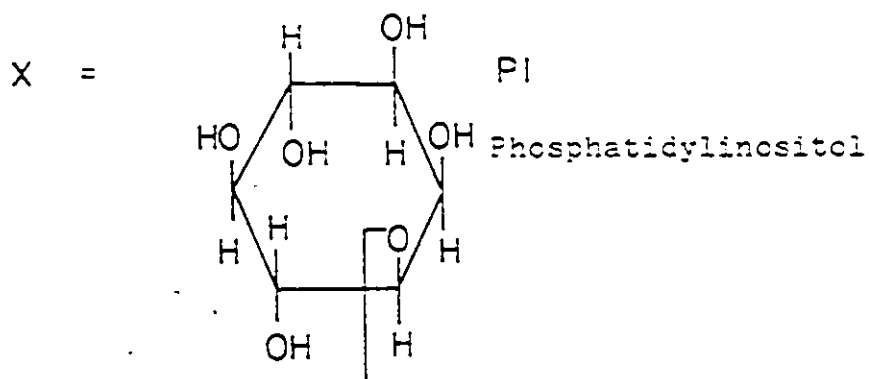
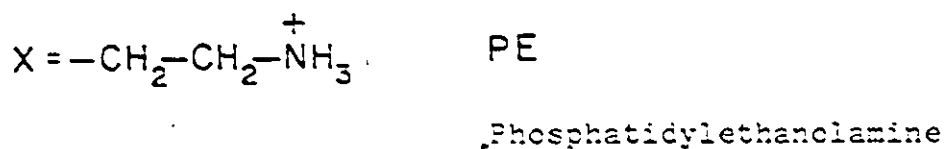
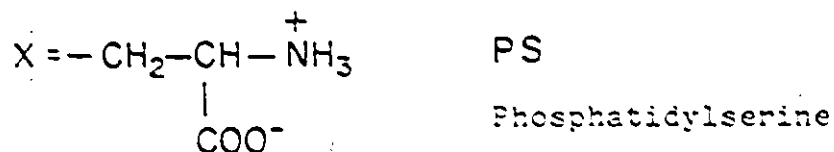
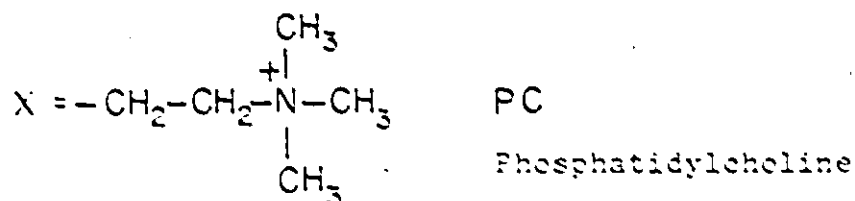


Fig. 3. Chemical structure of phospholipids (a) general structure and (b) different head group structures. The fatty acyl chain length (n) is variable and the chain can be saturated or unsaturated.

upon variation of hydration or temperature. Table 1 gives the properties of several natural and synthetic phospholipids.

(b) Local anesthetics

Anesthetics can be divided into two groups: general anesthetics and local anesthetics. General anesthetics act on the central nervous system whereas local anesthetics act locally on the membranes of the nerves. The dose required for analgesia is about ten times lower for general anesthesia (4 mmoles/kg membrane) than for local anesthesia (40 mmoles/kg membrane) (24).

The structure of general anesthetics are often very simple (xenon, nitrous oxide, chloroform, diethyl ether) while those of local anesthetics are more complex (25). Examples of local anesthetics are given in Table 2. Some molecules can be either local or general anesthetics depending on the concentration used and on the site of application.

In Fig. 4 are given the structures of local anesthetics of the aminoester and aminoamide types. Each molecule possesses three common regions: an amino

TABLE 1

Properties of some naturally-occurring
and synthetic phospholipids

Phospholipid.	Source.	T (°C) ^a	Major chain components	Ref.
PC	Egg yolk	-15--7	18:1, 16:0.	15-17
DPPC	Synthetic	41	16:0	15, 16
PS	Beef brain	0-10	18:0, 18:1	15, 16, 18-21
DPPS	Synthetic	53	16:0	15, 16, 20, 22
PE	Egg yolk	12-15	18:0, 16:0	15, 16, 23
PI	Wheat germ		18:0, 18:1	15, 16

^a T is the gel to liquid crystal phase transition temperature.

TABLE 2

Local Anesthetics

Class	Examples
n-Alkanols	Methanol Decanol
ω-Phenyl alkanols	Benzyl alcohol ω-Phenyl nonanol
Amines aminoesters aminoamides	Cocaine Procaine Lidocaine Tetracaine
Guanidine derivatives	Tetrodotoxin
Barbiturates	Pentobarbitone Phenobarbitone

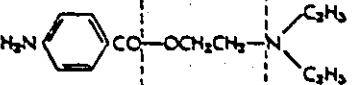
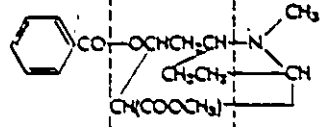
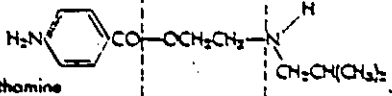
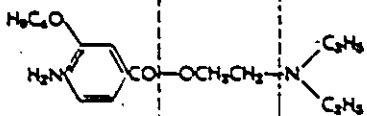
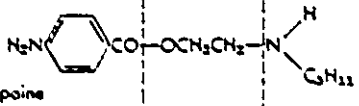
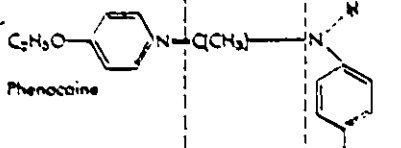
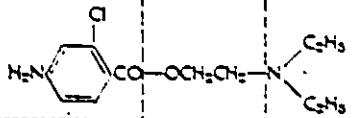
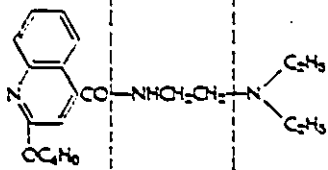
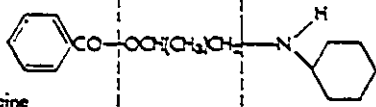
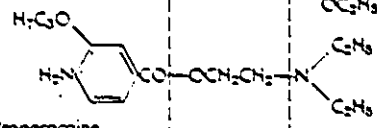
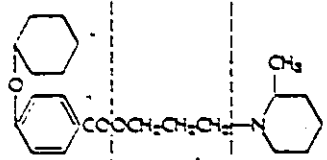
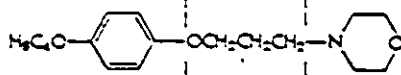
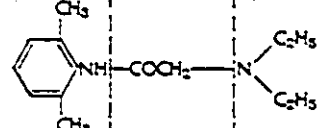
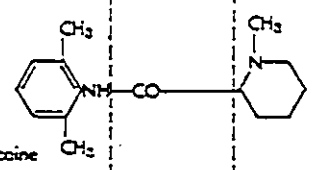
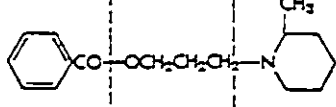
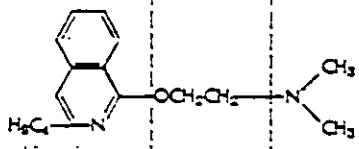
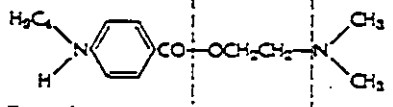
(AROMATIC RESIDUE)	(INTERMEDIATE CHAIN)	(AMINO GROUP)	(AROMATIC RESIDUE)	(INTERMEDIATE CHAIN)	(AMINO GROUP)
 Procaine	 Cocaine	 Bupivacaine	 Benzocaine	 Nasopaine	 Phenocaine
 Chloroprocaine	 Dibucaine	 Hexylcaine	 Propocaine	 Cyclomethacaine	 Premoxine
 Lidocaine	 Mepivacaine	 Piperocaine	 Dimethisoquin	 Tetracaine	

Fig. 4. Chemical structures of amine local anesthetics (26).

group, an aromatic residue and an intermediate chain (26). Local anesthetics of this type can exist in a charged and in an uncharged form. These amine-containing local anesthetics are also the most commonly used.

(c) Theories of anesthesia

Local anesthetics have been studied extensively in the recent years. In fact, numerous investigations have dealt with every possible aspect of these compounds. More specifically, about their mode of action, several review articles and books (25-42) now exist in spite of the fact that, up to the present, no clear mechanism has been demonstrated for their action.

It is possible to classify the literature about local anesthetics into three main classes of mechanisms of action: the receptor theory, the electrostatic theory and the lipid perturbation theory. First, the local anesthetic molecule can interact with a specific receptor in the nerve membrane. Such receptors have been proposed from kinetic and electrophysiological studies (43-45). This would explain why some tertiary amine-containing local anesthetics were found to be active in their charged form on the inside surface of the

nerve membrane (46). However, the receptor would have to be surprisingly unselective, or the number of different receptors extremely high, to account for the fact that such a wide range of compounds cause local anesthesia, their only obvious similarity being their general amphiphilic structure. Binding studies of anesthetics to proteins have often proven to be unrewarding (47). A recent study, however, postulated in the existence of a binding site for local anesthetics in nicotinic post-synaptic membranes (43).

A popular variant of this model is the sodium channel blocking effect (48). Interaction between the anesthetic and the sodium channel (protein) would induce a conformational change which would block the sodium ion passage. Unfortunately, the nature of the sodium channel, and of the anesthetic interaction with that site remains completely undefined.

A second proposal is that the effects of local anesthetics on the membrane surface charge would be responsible for their action (46). The fact that positively charged, negatively charged and uncharged anesthetics are all active cast some doubt about theories based solely on this interaction. Nonetheless, it is clear that when the local anesthetic acts in its charged

form, the electrostatic interaction must play an important role (49).

The last theory is concerned with the perturbation of the lipid structure of the membrane. Around the turn of the century, H. H. Meyer and E. Overton observed that the potency of an anesthetic was proportional to its solubility in olive oil. Subsequently, this was confirmed many times (50). The nerve conduction would then be blocked by a perturbation of the lipid part of the membrane that would alter in some way the conductivity process. This perturbation is either visualized as an expansion of the membrane lipid phase caused by the anesthetic (25), as a change in the gel to liquid crystal phase transition and phase separation of the lipids (51,52) or as a modification of the cooperative dynamic properties of the lipid molecules (53). These effects can be reversed by the use of high pressures (53). Such models would be best to account for the wide range of anesthetic agents. Phospholipids, which possess both a polar and an apolar part, and can be charged or neutral, are suitable for unspecific binding. It is also interesting to note that the effects of anesthetic mixtures are close to being additive, suggesting a single mode

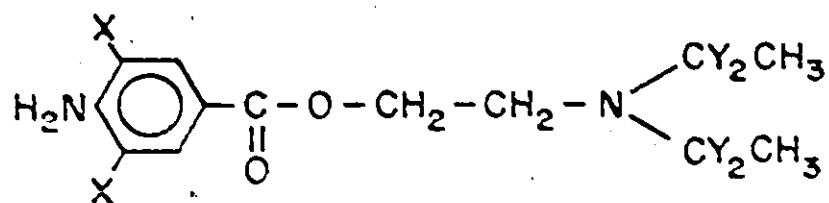
of action (54).

This theory was criticized by several authors on the basis of differences of potency between structurally related local anesthetics (41) and asymmetry of action across the membrane (55). Some studies also claimed that at concentrations necessary for anesthesia, the effects of local anesthetics on lipid membranes are very small (56) or non-existent (57), and concluded that the action of these agents must not be on lipids but on proteins. The fact remains that all anesthetics bind to membrane lipids: whether this interaction alone can explain the phenomenon of anesthesia, or whether it constitutes only a step in a chain of events is still unknown. Moreover, the location of the local anesthetic in the lipid membrane and the nature of the forces involved have not been precisely determined. It is the object of this thesis to explore anesthetic-membrane interaction in detail. Whatever the role of the lipids, a careful delineation of their interaction with anesthetics is necessary for complete understanding of anesthetic-membrane interaction.

B. Approach to the problem

This work is an investigation of the interaction between local anesthetics procaine (PBC) and tetracaine (TTC) and model membranes (multilamellar dispersions) composed of phosphatidylcholine (PC) and phosphatidylserine (PS) by deuterium (^2H) and phosphorus-31 (^{31}P) nuclear magnetic resonance (NMR). First, the local anesthetic-phospholipid behaviour was characterized under conditions suitable for NMR by determination of partition coefficients, pK_a values, solubility, extent of hydrolysis, variation in particle size... Both the local anesthetics (Fig. 5) and the phospholipid PC were then specifically-deuterated in several positions and studied by ^2H NMR at low and high pH values. ^{31}P NMR spectra of the phospholipid were also recorded. These studies allowed the characterization of the binding of the local anesthetic in its positively charged and uncharged forms with phospholipid multilamellar dispersions. A description of the techniques is given in the following sections.

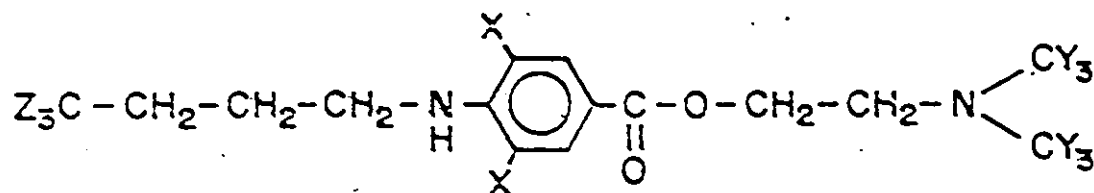
PROCAINE (PRC)



PRC-d₂, X = D, Y = H

PRC-d₄, X = H, Y = D

TETRACAINE (TTC)



TTC-d₂, X = D, Y = H, Z = H

TTC-d₃, X = H, Y = H, Z = D

TTC-d₆, X = H, Y = D, Z = H

Fig. 5. Chemical structure of local anesthetics procaine (PRC) and tetracaine (TTC) and their deuterated analogs.

C. Techniques

(a) Determination of partition coefficients

Partition coefficients or distribution coefficients are defined as the ratio of the concentrations (activities) of a solute in two immiscible solvents (58). This definition applies to a lipid dispersion where the solute is the local anesthetic and the solvents are the aqueous phase and the phospholipid multilamellar dispersions (59). Partition coefficients K_p can be expressed as:

$$K_p = \frac{[A_l]}{[A_w]} = \frac{A_l/M_l}{A_w/M_w} \quad (1)$$

where $[A_l]$ and $[A_w]$ are the concentrations of anesthetic in the lipid and water phases, respectively, at equilibrium (expressed in grams of anesthetic per gram of phase), A_l and A_w are the weights of the anesthetic in each phase and, M_l and M_w , the weights of each phase ^{assumed} (the density of the phospholipid to be 1.00 g/ml).

Knowing the amount of water and of phospholipid and the total concentration of local anesthetic, a measurement of the aqueous local anesthetic concentration by UV-VIS spectroscopy allows the determination

of the partition coefficient (see Section III.D.a).

(b) Electron spin resonance

Electron spin resonance (ESR) is a technique widely used that allows the determination of molecular order and correlation times in biological and model membranes (11, 60, 61). Basically, the technique is similar to nuclear magnetic resonance (NMR) except that it observes the interaction of an unpaired electron, rather than a nucleus, with a magnetic field. The interaction can be described by the Hamiltonian (62):

$$\mathcal{H} = g\mu_B \hat{S} + \hat{I} \cdot \bar{A} \cdot \hat{S} + \mathcal{H}_{\text{exch}} + \mathcal{H}_{\text{dipolar}} \quad (2)$$

where the first term corresponds to the Zeeman interaction between the magnetic field $\bar{H}$ and the electron spin $\hat{S}$. g is the g-factor of the electron, and μ_B is the Bohr magneton. The second term describes the coupling between the nuclear spin $\hat{I}$ and the electron spin $\hat{S}$, which is anisotropic; $\bar{A}$ is the coupling constant tensor. $\mathcal{H}_{\text{exch}}$ and $\mathcal{H}_{\text{dipolar}}$ are the exchange and dipolar contributions to the Hamiltonian.

In the spectrum of a nitroxide radical ($>\text{N-O}^\bullet$) which has a free electron on the oxygen coupled to a spin-1 nitrogen nucleus, three transitions are allowed

due to the hyperfine coupling between them. The width of the coupling depends on the orientation of the nitrogen π -orbital relative to the magnetic field. In isotropic solution, the coupling constant a corresponds to the average value of the diagonal elements of the $\bar{A}$ tensor i.e. $a = 1/3 (2A_{\perp} + A_{\parallel})$ where $A_{\parallel}$ (32 G) and $A_{\perp}$ (6 G) are the coupling constant values when the nitrogen π -orbital is parallel and perpendicular to the magnetic field, respectively.

In a typical ESR experiment, the concentration of spin label probes is usually very low (10^{-5} M): when it is increased, two other interactions start to play a role: the electron-electron dipolar interaction and the electron-electron spin exchange interaction (61). The dipolar interaction is analogous to the classical magnetic interaction between two bar magnets and is inversely proportional to the cube of the distance between the spin labels. This provides a possible method for measuring distances between spin-labeled molecules.

The spin exchange interaction is a quantum mechanical effect which requires contact between the spin labeled molecules. A biradical state of very short lifetime is formed, resulting in a broadening of the

ESR resonances. The peak-to-peak linewidth, ΔH_{ex} , of the central line in the first derivative spectrum is given by (61):

$$\Delta H_{ex} = 2 \left(\frac{h}{g\beta} \right) W_{ex} \quad (3)$$

where h is Planck's constant and W_{ex} is the exchange frequency (frequency of collision between spin labels).

In this work, the spin exchange interaction has been used to monitor the penetration of the local anesthetic molecule into the lipid multilamellar dispersion. As the local anesthetic intercalates into the bilayers, the distance between spin labeled phospholipid molecules increases i.e. their frequency of collision, the spin exchange interaction decreases and the ESR resonances become sharper (see Section IV.B.f).

(c) ^2H nuclear magnetic resonance

i. Principles

The ^2H NMR technique has considerably developed in the last few years and is now used mostly in systems where ^1H and ^{13}C NMR are difficult or impossible (solids, liquid crystals, biological membranes, etc). The theory

concerning ^2H NMR (63-65) and its applications to membranes (65-67) has been reviewed.

Deuterium is a relatively rare isotope of hydrogen: its natural abundance is 0.015%. Its sensitivity relative to hydrogen is 9.65×10^{-3} and it has a spin $I = 1$. It is observed at an NMR frequency equivalent to 15.351% of the proton frequency. These considerations seem to make ^2H an unlikely candidate for NMR investigation. In fact, several means can be used to improve these conditions. First, compounds can be enriched by chemical synthesis. Second, high magnetic fields can be used to increase the spin population difference i.e. the sensitivity. Using a sequence of pulses, the magnetization can be refocussed to avoid losses due to electronic limitations (quadrupole echo technique, see Section iv). Finally, concentration, sample tube diameter and observing parameters can be optimized to attain the maximum signal to noise in the NMR spectrum (68).

In isotropic solutions, ^2H NMR can be used in a similar way to ^1H or ^{13}C NMR (63, 66). The spectral width is only 154 Hz (at 23 kG) and the spin-spin coupling constants are reduced by a factor of 6.5 compared to proton (the magnetogyric ratios $\gamma_{\text{H}} = 6.5 \gamma_{^2\text{H}}$). The

main interest of this study is not to observe molecules in isotropic solvent but rather to observe them in a liquid crystal environment where the quadrupolar splitting is not reduced to zero.

In a motion^{ally} restricted system, the energy of a deuterium nucleus in a magnetic field is composed of the magnetic energy E_M and the quadrupole energy E_Q . The total Hamiltonian $\mathcal{H}$ can be written (65):

$$\mathcal{H} = \mathcal{H}_M + \mathcal{H}_Q \quad (4)$$

where $\mathcal{H}_M$ is the magnetic Hamiltonian describing the interaction of the nuclear magnetic moment μ_N with the magnetic field H_0

$$\mathcal{H}_M = -\mu_N H_0 = -g s_N \hat{I} \cdot H_0 \quad (5)$$

$\hat{I}$ is the nuclear spin operator, s_N the nuclear magneton and g the so-called g factor. The quadrupole Hamiltonian $\mathcal{H}_Q$ arises from the electrostatic interaction between the nuclear quadrupole moment (Q_{ik}) and the electric field gradient $\nabla E = V_{ik}$ at the nucleus. The quadrupole Hamiltonian can be formulated (68):

$$\mathcal{H}_Q = \frac{eQ}{4I(2I-1)} [\bar{V}_0(3\hat{I}_z^2 - \hat{I}^2) + \bar{V}_{\pm 1}(\hat{I}_+ \hat{I}_z + \hat{I}_z \hat{I}_+) + \bar{V}_{\pm 2}\hat{I}_+^2] \quad (6)$$

e is the elementary charge, Q is the quadrupole moment and $\hat{I}$ is the nuclear spin operator. The other terms are defined as:

$$\begin{aligned} \bar{V}_0 &= \bar{V}_{zz}, \\ \bar{V}_{\pm 1} &= \bar{V}_{xz} \pm i\bar{V}_{yz}, \\ \bar{V}_{\pm 2} &= \frac{1}{2} (\bar{V}_{xx} - \bar{V}_{yy} \pm 2i\bar{V}_{xy}) \end{aligned} \quad (7)$$

The subscripts x , y and z indicate second derivatives of the electrostatic potential V with respect to the corresponding axes. $\hat{I}_+$ and $\hat{I}_-$ are the raising and lowering operators:

$$\hat{I}_{\pm} = \hat{I}_x \pm i\hat{I}_y \quad (8)$$

Solution of the Hamiltonian yields three energy values (E_{+1} , E_0 , E_{-1}) between which two transitions are possible ($\Delta m_I = \pm 1$). The difference between the two transition peaks is called quadrupole splitting, D_Q , and is angular dependent. For $V_{xx} = V_{yy}$ (the asymmetry parameter $\eta = 0$), the quadrupole splitting is given by:

$$D_Q(\theta) = \frac{3}{4} \left(\frac{e^2 q Q}{h} \right) (3 \cos^2 \theta - 1) \quad (9)$$

where ($e^2 qQ/h$) is the quadrupole coupling constant and θ is the angle between the axis of V_{zz} and the applied magnetic field. It is interesting to note that the quadrupole splitting collapses at $\theta = 54.74^\circ$ (magic angle).

If, in a sample all orientations of V_{zz} are equally probable, as in a polycrystalline sample, there will be a continuum of doublets covering all values of θ . The case of a multilamellar lipid dispersion is illustrated in Fig. 6. This envelope which has a maximum at $\theta = 90^\circ$ has a shape defined by:

$$p(\theta) = \frac{1}{2} \sin \theta \quad (10)$$

and a quadrupole splitting at maximum intensity:

$$D_Q (\theta = 90^\circ) = \left| \frac{3}{4} \left(\frac{e^2 qQ}{h} \right) \right| \quad (11)$$

In subsequent discussions, the term "quadrupole splitting" will refer to that for the angle $\theta = 90^\circ$.

In liquid crystals, molecules are not static but fluctuate anisotropically in the sample. The average over this fluctuation is expressed by the order parameter S_{CD} :

$$S_{CD} = \frac{1}{2} \langle 3 \cos^2 \theta(t) - 1 \rangle \quad (12)$$

ORIGIN OF ^2H POWDER SPECTRUM

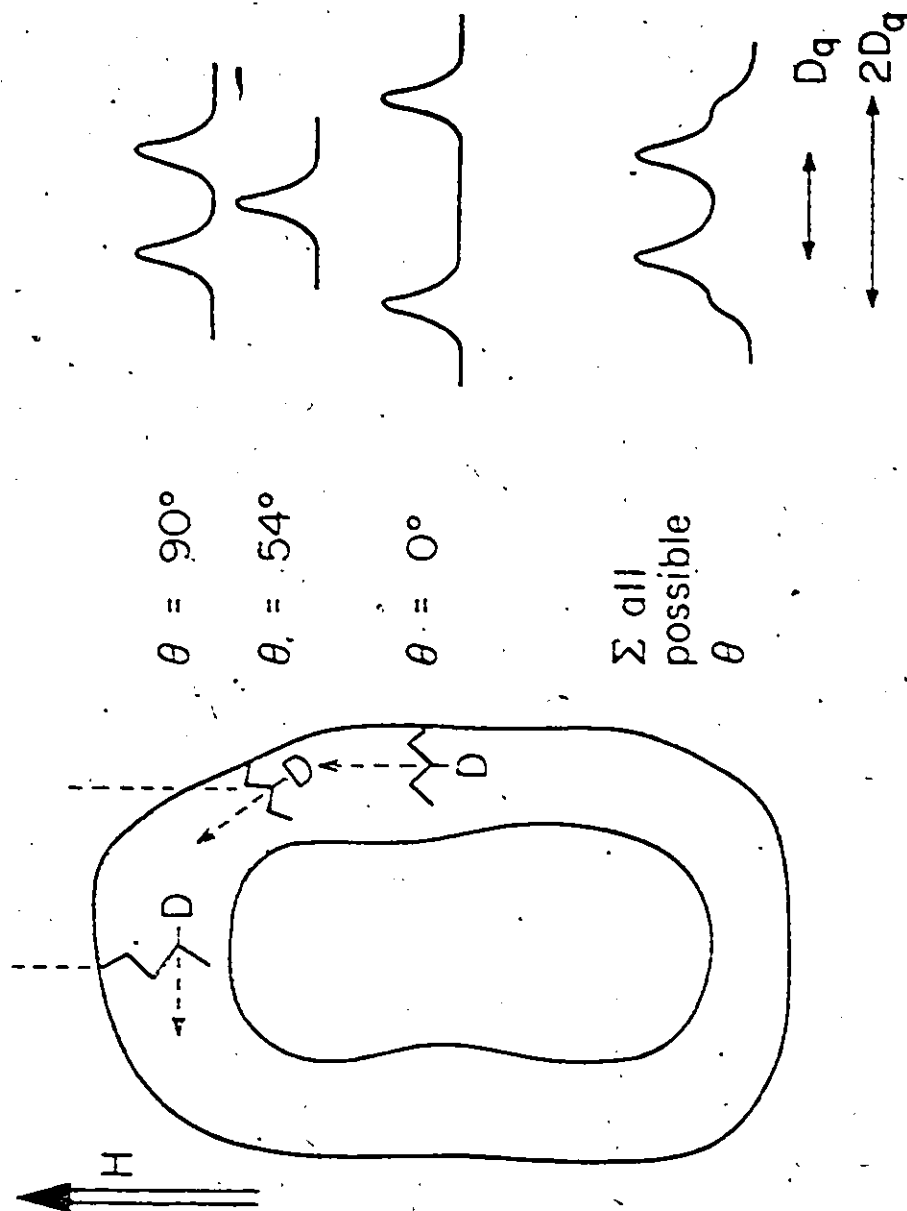


Fig. 6. Representation of ^2H NMR doublets observed at different angles θ between the magnetic field H and the C-D bond in a lamellar dispersion. The summation of these doublets gives rise to the powder pattern spectrum.

where $s(t)$ is the time varying angle between the major axis of the electric field gradient and the director (Fig. 7) and the brackets represent a time or ensemble average (69). Thus, in multilamellar dispersions or any similar systems, the quadrupole splitting of Eq. 11 is effectively reduced by a factor S_{CD} :

$$D_Q = \frac{3}{4} \left(\frac{e^2 q Q}{h} \right) S_{CD} \quad (13)$$

This equation is fundamental to the interpretation of all quadrupole splittings observed in this work.

ii. Molecular order parameters

The order parameters of different deuterons in the same molecule can be related by converting the order parameter along the C-D bond S_{CD} into a molecular order parameter S_{mol} along the director axis. For a methylene group (CD_2), S_{mol} is perpendicular to the plane passing through the CD_2 atoms and for a terminal methyl S_{mol} is in the direction of the carbon-carbon bond, in an aliphatic saturated chain (70). With these directions in mind, the molecular order parameter becomes (69, Fig. 8):

MOLECULAR ORDER PARAMETERS

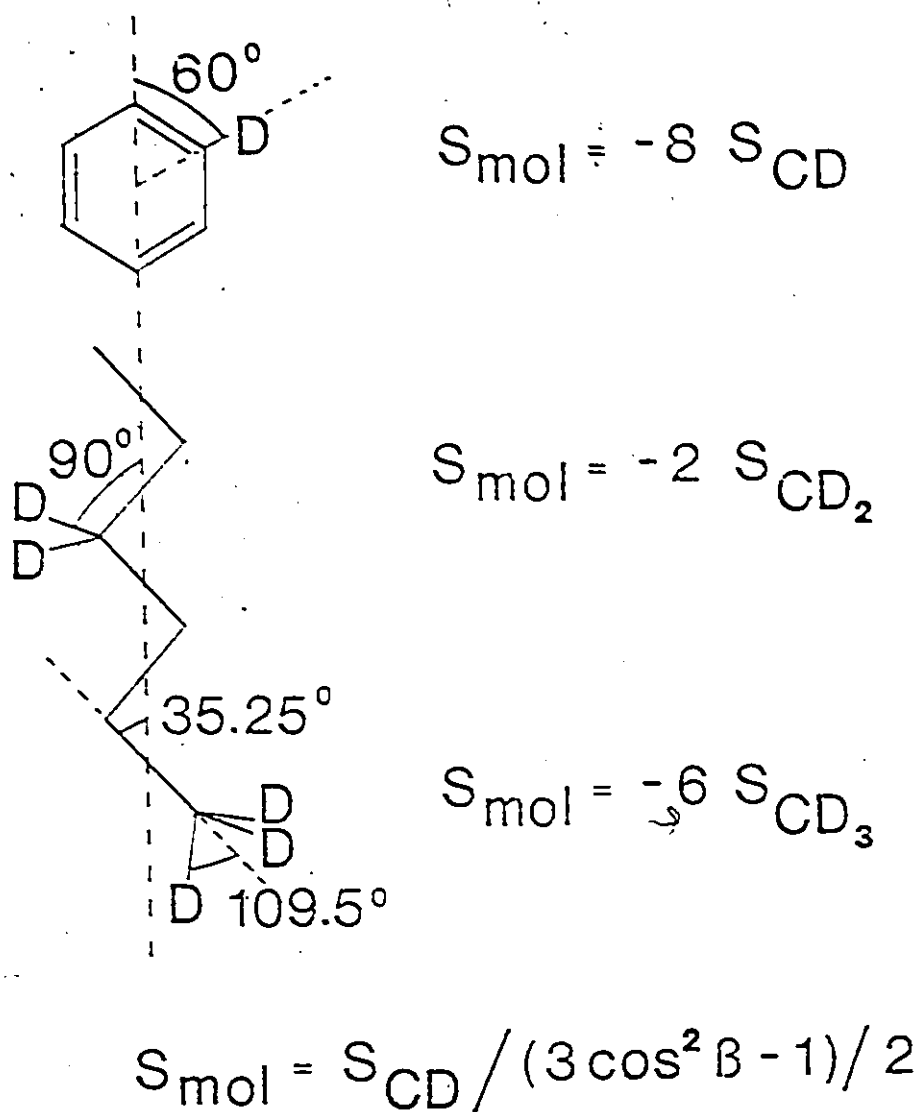


Fig. 8. Conversion of order parameters along the C-D bond S_{CD} into molecular order parameters along the director axis S_{mol} for an aromatic C-D bond and aliphatic methylene and methyl bonds.

$$S_{\text{mol}} = S_{\text{CD}_2} / \left[\frac{1}{2} (3\cos^2 90^\circ - 1) \right] = -2S_{\text{CD}_2} \quad (14)$$

$$S_{\text{mol}} = S_{\text{CD}_3} / \left[\frac{1}{2} (3\cos^2 109.5^\circ - 1) \right] \times \frac{1}{2} (3\cos^2 35.25^\circ - 1) = -6S_{\text{CD}_3} \quad (15)$$

In the case of an aromatic nucleus, where the director axis would pass through the para substituents, S_{mol} for positions 2, 3, 5 and 6 is given by:

$$S_{\text{mol}} = S_{\text{CD}} / \left[\frac{1}{2} (3\cos^2 60^\circ - 1) \right] = -8S_{\text{CD}} \quad (16)$$

iii. Effect of exchange on ^2H NMR spectra

The NMR spectrum of a species can be modified by the exchange of that species between two states or environments (70-75). The spectrum depends on whether the rate of exchange is fast or slow on the NMR time-scale. For the case of a species exchanging between two phases, one of which gives rise to a quadrupole splitting and the other to a single resonance, the exchange will be slow if the lifetimes τ of the observed

species (residence time) in the two individual sites are much longer than the inverse of the difference between the quadrupole splitting D_Q and the linewidth $\Delta\nu_{1/2}$.*

$$\tau \gg \frac{1}{D_Q - \Delta\nu_{1/2}} = \frac{1}{D_Q} \quad (17)$$

and two separate signals will be observed. If the lifetime τ is much shorter than $1/D_Q$, then an average signal $\Delta\nu$ (quadrupole splitting or broadened line) will result and the species will be in fast exchange:

$$\Delta\nu = x_1 D_Q + x_2 \Delta\nu_{1/2} \quad (18)$$

x_1 and x_2 being the mole fractions in sites 1 and 2, respectively. For the intermediate case, a more complicated lineshape is expected (see Section II.B).

Several examples of NMR spectra in the presence of exchange for quadrupolar nuclei have been published (71-74).

iv. Quadrupole echo technique

Conventional Fourier transform (FT) NMR consists in applying a single pulse (usually 90°) to a sample

* The word linewidth refers to a representative quadrupole splitting giving rise to a line having such a width arising from a distribution or superposition of quadrupole splittings.

in a magnetic field under conditions that would tip the magnetization in the rotating frame by the chosen angle (68°). Immediately after the pulse, the magnetization recovers towards the initial equilibrium conditions and the decay of the magnetization in the y-axis (M_y) is recorded as a function of time. The signal, called free induction decay (FID), varies according to the transverse relaxation time T_2 (Fig. 9a):

$$\frac{dM_y}{dt} = -\frac{M_y}{T_2} \quad (19)$$

The FID is then Fourier transformed from the time domain into the frequency domain to give the NMR spectrum:

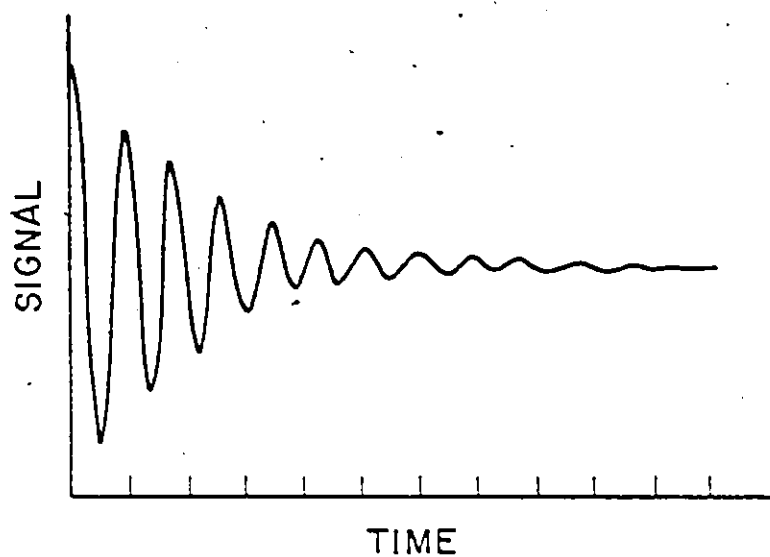
$$F(\nu) = \int_{-\infty}^{\infty} f(t) \exp(i2\pi\nu t) dt \quad (20)$$

where $F(\nu)$ is the frequency function and $f(t)$ is the time function.

When the linewidth becomes very broad, as in the case of ^2H NMR quadrupole splittings (liquid crystals, solids), the value of T_2 is very short and the decay of the magnetization very fast.* Since the dead-time (time period after the pulse before ^{data} acquisition due to electronic limitations) of most spectrometers

* Does not apply to the case where the linewidth is the result of the superposition of small quadrupole splittings.

(a)



(b)

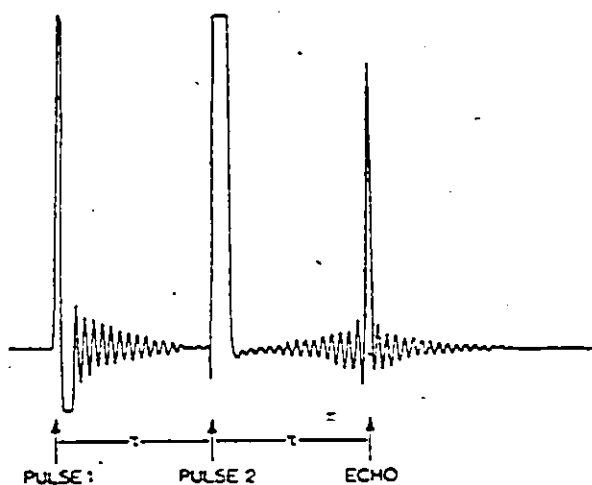


Fig. 9. Typical free induction decay (FID) obtained after (a) a 90° pulse and (b) a $90^\circ-\tau-90^\circ-\tau$ -Echo i.e. a quadrupole echo pulse sequence $x(76).y$

is several tens of microseconds, much of the information about these broad components may be lost (76). The accumulation of the spectrum becomes very time-consuming or impossible. As an alternative, the quadrupole spin-echo technique has been developed (76). The pulse sequence used in this technique is $90^\circ_X - \tau - 90^\circ_Y - \tau - \text{Echo}$. After a time τ , a second 90° pulse is applied but in a direction perpendicular to the first pulse. A complete refocussing of the nuclear magnetization called a "quadrupole spin-echo" occurs at time $t = 2\tau$ (Fig. 9b). Transformation of the signal starting at the top of the echo yields the complete spectrum aside from magnetic field inhomogeneities and relaxation effects.

Distortions in the ^2H NMR lineshape may also arise from excessively long 90° pulses which results in an unequal distribution of the rf power across the spectrum. As an example, for a 25 kHz quadrupole splitting, a pulse length below 5 μsec should give rise only to minor distortions in the outer wings of the quadrupole splitting pattern.

(d) ^{31}P nuclear magnetic resonance

Phosphorus-31 is a naturally abundant (100%) spin-1/2 nucleus whose NMR spectra, in anisotropic

media, are affected by chemical shift anisotropy. The nuclear Zeeman Hamiltonian can be represented by (77):

$$\mathcal{H} = \gamma_N \hbar \gamma_N H_0 (1 - \sigma) \hat{I}_z \quad (21)$$

where γ_N is the nuclear g factor, $\hbar \gamma_N$ the nuclear magneton, H_0 the magnetic field, σ the chemical shift tensor and $\hat{I}_z$ the nuclear spin operator in the z-direction. Solving the Hamiltonian for an oriented sample, the angular dependent frequency $\nu(\theta)$ of the resonance has a value:

$$\nu(\theta) = \frac{\gamma_N \hbar \gamma_N H_0}{h} \left(1 - \sigma_i - \frac{2}{3} \Delta\sigma \left(\frac{3\cos^2\theta - 1}{2} \right) \right) \quad (22)$$

where σ_i is the isotropic chemical shift value, $\Delta\sigma$ is the difference between the chemical shift tensors when the magnetic field is parallel ($\sigma_{||}$) and perpendicular ($\sigma_{\perp}$) to the director axis (possible because of fast averaging about the director axis), and θ is the angle between the magnetic field and the director axis.

In a multilamellar phospholipid dispersion, a powder spectrum is observed, resulting from the sum of the resonances at all angles θ (Fig. 10). The difference between the chemical shift tensors $\Delta\sigma = \sigma'_{||} - \sigma'_{\perp}$ is a measure of the degree of order experienced by the

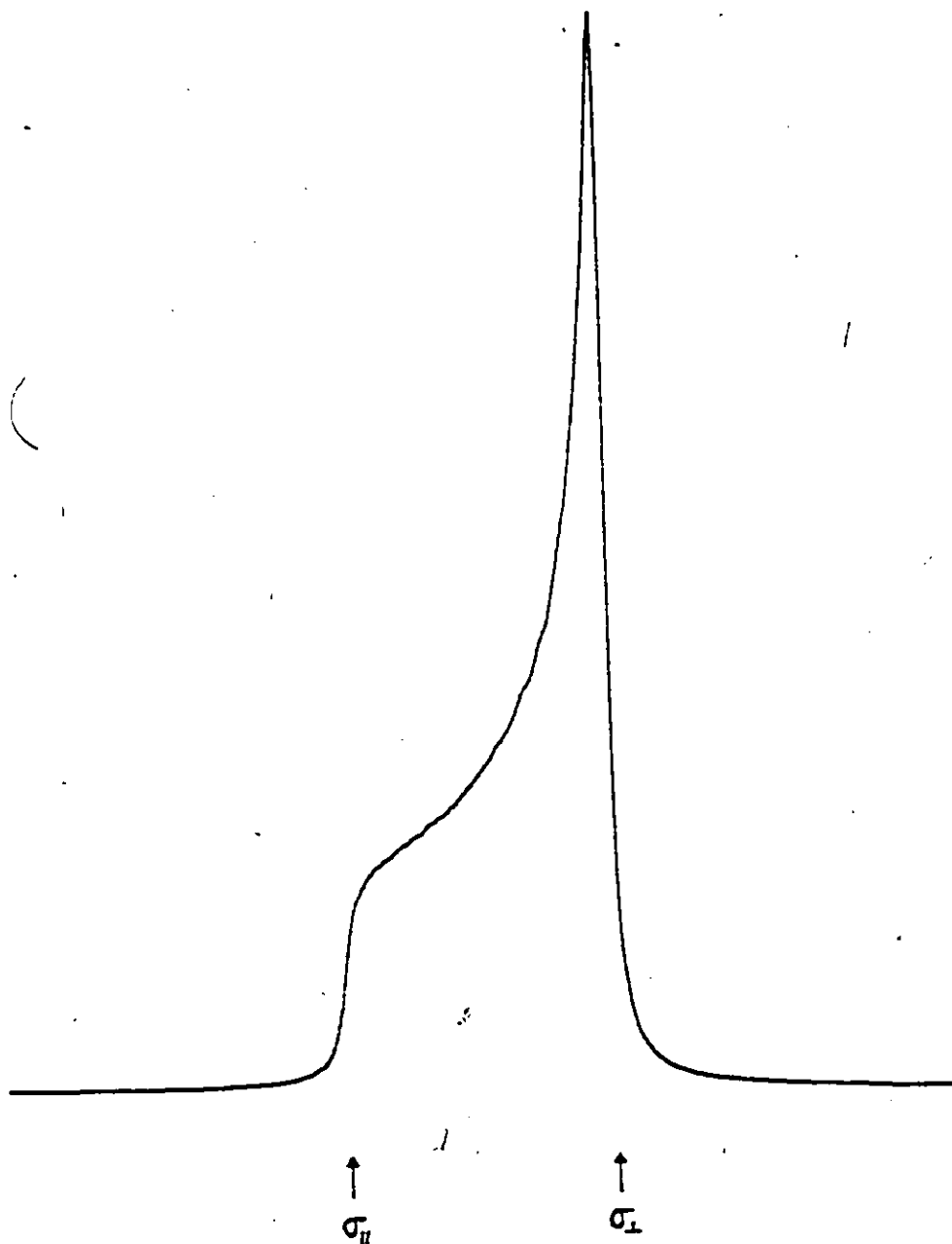


Fig. 10. Theoretical ^{31}P NMR powder pattern for a bilayer type of dispersion. The two chemical shift tensors $\sigma_{\parallel}$ and $\sigma_{\perp}$ correspond to the left and to the right of the powder pattern, respectively.

phosphorus nucleus:

$$\Delta\sigma = (\sigma_{33} - \sigma_{22})S_{33} \quad (23)$$

The orientation of the chemical shift tensors σ_{22} and σ_{33} is illustrated in Fig. 11 for a phosphate segment in a phospholipid molecule. S_{33} is the order parameter for the direction defined in Fig. 11. However, a change in $\Delta\sigma$ is not necessarily due to a change in order; a perturbation in phosphate symmetry may affect the gradient tensors with no correlation with the degree of order (77).

In practice, ^{31}P NMR spectra are not normally used to monitor order (due to the difficulty in differentiating between order and symmetry effects) but to probe phase changes from bilayer to hexagonal where the values of $\Delta\sigma$ is reduced by a factor $-1/2$ (78). Thus, a clear difference in the shape of the spectrum is observable.

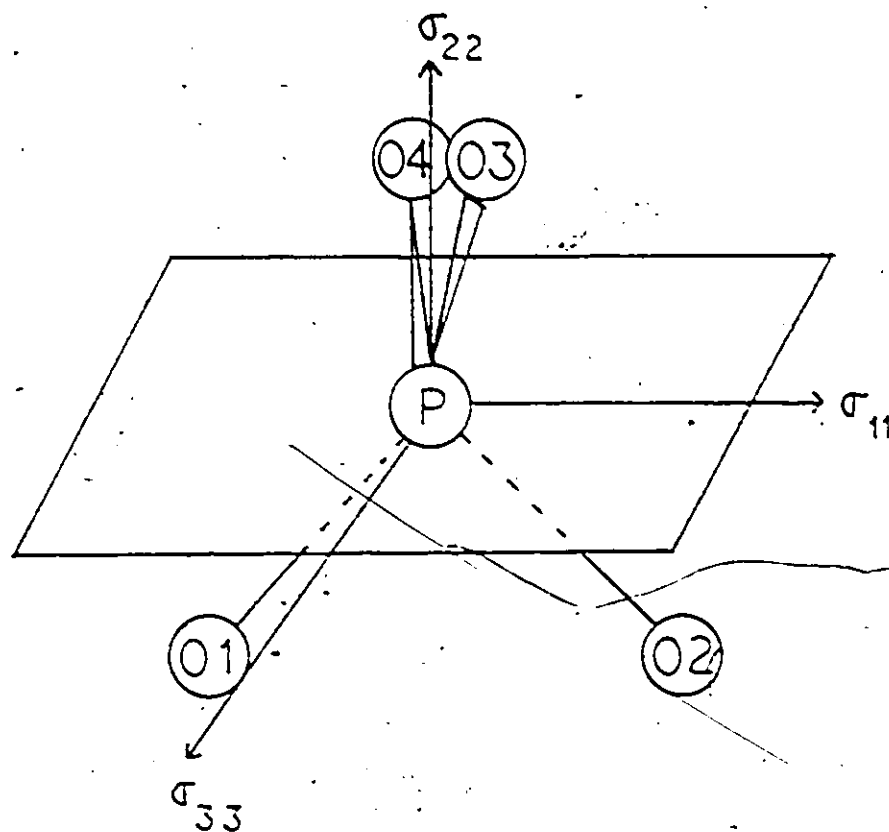
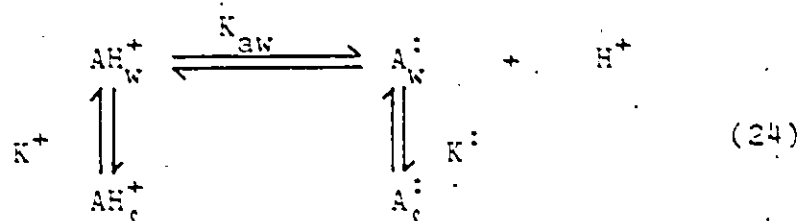


Fig. 11. Orientation of the ^{31}P chemical shift tensors σ_{ii} for the phosphate group. O(3) and O(4) are the two nonesterified oxygens. O(1) and O(2) connect the phosphate group to the glycerol backbone and the head group residue, respectively, of the phospholipid molecule (77).

II. THEORETICAL DERIVATIONS

A. Calculation of amount of local anesthetic in each form and in each phase

The different forms in which the local anesthetic can find itself are represented by the following scheme:



AH_w^+ and AH_l^+ are the positively charged forms of the local anesthetic in the water and lipid phases, respectively, and $\text{A}_w \cdot$ and $\text{A}_l \cdot$ are the uncharged forms of the same local anesthetic in the water and lipid phases, respectively. The constants K^+ and $\text{K}^{\cdot}$ are the partition coefficients of each form which are defined as:

$$\text{K}^+ = \frac{[\text{AH}_l^+]}{[\text{AH}_w^+]}, \quad \text{K}^{\cdot} = \frac{[\text{A}_l \cdot]}{[\text{A}_w \cdot]} \quad (25)$$

where $[\text{AH}_l^+]$ and $[\text{A}_l \cdot]$ are the total concentration of charged and uncharged local anesthetic in the lipid phase and $[\text{AH}_w^+]$ and $[\text{A}_w \cdot]$ the concentration of charged and uncharged local anesthetic in the water phase, res-

pectively. The experimental partition coefficient K_p is given by (assuming that K^+ and $K^\cdot$ are concentration independent):

$$K_p = x_{AH^+} K^+ + x_A K^\cdot \quad (26)$$

where x_{AH^+} and x_A are the mole fractions of the charged and uncharged local anesthetic, respectively. The constant K_{aw} is defined as:

$$K_{aw} = \frac{[A^\cdot_w][H^+]}{[AH^+_w]} \quad (27)$$

and its negative logarithm corresponds to the pK_a value.

Knowing the pK_a value of the local anesthetic and the partition coefficients at two different pH values, the amount of local anesthetic in each form can be calculated. Considering two pH values, pH_1 and pH_2 , the partition coefficients for the charged and uncharged species are given by:

$$\begin{aligned} K_1^+ &= \frac{[AH^+_{l1}]}{[AH^+_{w1}]} & K_1^\cdot &= \frac{[A^\cdot_{l1}]}{[A^\cdot_{w1}]} \\ K_2^+ &= \frac{[AH^+_{l2}]}{[AH^+_{w2}]} & K_2^\cdot &= \frac{[A^\cdot_{l2}]}{[A^\cdot_{w2}]} \end{aligned} \quad (28)$$

The total concentrations in the lipid phase at each pH, $[A_{t1}]$ and $[A_{t2}]$ are:

$$[A_{t1}] = [A_{l1}] + [AH_{l1}^+] = K^-[A_{w1}] + K^+[AH_{w1}^+]^* \quad (29)$$

$$[A_{t2}] = [A_{l2}] + [AH_{l2}^+] = K^-[A_{w2}] + K^+[AH_{w2}^+]^* \quad (30)$$

From Eq. 29,

$$K^- = \frac{[A_{l1}] + [AH_{l1}^+] - K^+[AH_{w1}^+]}{[A_{w1}]} \quad (31)$$

Introducing into Eq. 30:

$$[A_{l2}] + [AH_{l2}^+] = \left(\frac{[A_{l1}] + [AH_{l1}^+] - K^+[AH_{w1}^+]}{[A_{w1}]} \right) [A_{w2}] + K^+[AH_{w2}^+] \quad (32)$$

and rearranging:

$$K^+ = \frac{[A_{w1}][A_{t2}] - [A_{w2}][A_{t1}]}{[AH_{w2}^+][A_{w1}] - [AH_{w1}^+][A_{w2}]} \quad (33)$$

Similarly, the partition coefficient for the uncharged form K^- , can be obtained:

$$K^- = \frac{[AH_{w1}^+][A_{t2}] - [AH_{w2}^+][A_{t1}]}{[AH_{w2}^+][A_{w1}] - [AH_{w1}^+][A_{w2}]} \quad (34)$$

* K^+ and K^- are assumed to be independent of pH.

From the values of the partition coefficients K^+ and K^- , it is possible to calculate the amount of local anesthetic in each form and in each phase. The total amount of local anesthetic A_t in the system is:

$$A_t = A_w^- + A_l^- + AH_w^+ + AH_l^+ \quad (35)$$

Using Eqs. 25 and 27, this equation can be transformed to:

$$A_t = A_w^- \left(1 + \frac{K^- V_l}{V_w} + \alpha + \frac{\alpha K^+ V_l}{V_w} \right) \quad (36)$$

where $\alpha = 10^{pK_a - pH}$ and V_l and V_w are the volumes of the lipid and water phases, respectively (corresponding to M_l and M_w). This yields:

$$A_w^- = \frac{A_t V_w}{V_w(1 + \alpha) + V_l(K^- + \alpha K^+)} \quad (37)$$

$$A_l^- = \frac{K^- A_w^- V_l}{V_w}$$

$$AH_w^+ = \alpha A_w^-$$

$$AH_l^+ = \frac{K^+ AH_w^+ V_l}{V_w}$$

and the amount of local anesthetic in each form and in each phase can be calculated.

B. Calculation of partition coefficient from ^2H NMR variable linewidth

In the case of fast exchange of a local anesthetic molecule between the lipid and water phases, the observed ^2H NMR linewidth or quadrupole splitting $\Delta\nu_{\text{obs}}$ is expressed as (Eq. 18):

$$\Delta\nu_{\text{obs}} = (1 - x_l)\Delta\nu_w + x_l\Delta\nu_l \quad (38)$$

where $\Delta\nu_w$ and $\Delta\nu_l$ are the values of linewidth or quadrupole splitting in absence of exchange in the water and lipid phases, respectively, and x_l is the mole fraction of local anesthetic in the lipid phase. Expressing in terms of amount of local anesthetic and assuming only one form of local anesthetic, Eq. 38 becomes:

$$\Delta\nu_{\text{obs}} = \frac{A_l}{A_t} \Delta\nu_w + \frac{A_w}{A_t} \Delta\nu_l \quad (39)$$

where A_l and A_w are the amounts of local anesthetic in the lipid and water phases, respectively, and A_t the total amount of local anesthetic.

The partition coefficient K_p (Eq. 1) is related to those amounts:

$$K_p = \frac{A_l V_w}{A_w V_l} \quad (40)$$

and rearranging:

$$A_l = A_t - A_w = \frac{K_p V_l A_w}{V_w} \quad (41)$$

$$A_w = \frac{A_t V_w}{K_p V_l + V_w} \quad (42)$$

Introducing those two equations into Eq. 39, the following expression is obtained:

$$V_w = K_p V_l (\Delta v_l - \Delta v_w) \frac{1}{\Delta v_{obs} - \Delta v_w} - K_p V_l \quad (43)$$

which can be used when the volume of the water phase is varied. A plot of V_w vs $1/(\Delta v_{obs} - \Delta v_w)$ allows the determination of the partition coefficient (intercept) and of the linewidth of the lipid bound species Δv_l (slope). A similar expression can be used when the amount of lipid phase is varied:

$$\frac{1}{V_l} = \frac{K_p}{V_w} (\Delta v_l - \Delta v_w) \frac{1}{\Delta v_{obs} - \Delta v_w} - \frac{K_p}{V_w} \quad (44)$$

C. Calculation of ^2H NMR spectra in the presence of exchange

Using modified Bloch equations (79), expressions have been derived to calculate NMR spectra in the presence of exchange between two sites whose transverse relaxation times T_2 are equal (80,81). This theory has been modified here for sites having different values of T_2 and used to calculate spectra resulting from exchange between a quadrupolar powder pattern and a single resonance.

The magnetization in the x-y plane of species A and B is given as $M_A = u_A + iv_A$ and $M_B = u_B + iv_B$ where u and v are the two components of nuclear magnetization in directions perpendicular to the magnetic field (81). From the modified Bloch equations (79), M_A and M_B satisfy the equations (no saturation):

$$\begin{aligned} \frac{dM_A}{dt} + \left[\frac{1}{T_{2A}} - i \left(\Delta\omega + \frac{\delta\omega}{2} \right) \right] M_A &= -i\omega_1 M_0 \\ \frac{dM_B}{dt} + \left[\frac{1}{T_{2B}} - i \left(\Delta\omega - \frac{\delta\omega}{2} \right) \right] M_B &= -i\omega_1 M_0 \end{aligned} \quad (45)$$

where T_{2A} and T_{2B} are the transverse relaxation times of the species in site A and B, respectively, $\Delta\omega$ is the difference between the average frequency of the two

species in absence of exchange and the observing frequency:

$$\Delta\omega = \left(\frac{\omega_A + \omega_B}{2} \right) - \omega \quad (46)$$

$\delta\omega$ is the difference in frequency between the two species in absence of exchange ($\omega_A - \omega_B$). ω_1 is ^{the amplitude of} the applied radiofrequency ^{field} and M_0 the original magnetization (z-direction).

These equations were solved following the method of Gutowsky and Holm (80) to yield the total magnetization M in the presence of exchange:

$$M = \frac{i\omega_1 M_0 (\tau_A + \tau_B) + \tau_A \tau_B (\alpha_A \rho_B + \alpha_B \rho_A)}{(1 + \alpha_A \tau_A)(1 + \alpha_B \tau_B) - 1} \quad (47)$$

where $\alpha_A = 1/T_{2A} - i(\Delta\omega + \delta\omega/2)$ and $\alpha_B = 1/T_{2B} - i(\Delta\omega - \delta\omega/2)$, τ_A and τ_B are the residence times A and B, respectively, ρ_A and ρ_B the fractions of A and B, respectively:

$$\rho_A = \frac{\tau_A}{\tau_A + \tau_B}, \quad \rho_B = \frac{\tau_B}{\tau_A + \tau_B} \quad (48)$$

Expanding Eq. 47 and dividing both the numerator N and the denominator D by $\tau_A + \tau_B$, we obtain:

$$N = 1 + \tau \left(\frac{p_B}{T_{2A}} + \frac{p_A}{T_{2B}} \right) - i \left(\tau \left(\Delta\omega - ((p_A - p_B) \frac{\delta\omega}{2}) \right) \right) \quad (49)$$

$$D = \frac{p_A}{T_{2A}} + \frac{p_B}{T_{2B}} + \tau \left(\frac{1}{T_{2A}T_{2B}} - \Delta\omega^2 + \left(\frac{\delta\omega}{2} \right)^2 \right) - i \left[\Delta\omega \left(1 + \tau \left(\frac{1}{T_{2A}} + \frac{1}{T_{2B}} \right) \right) + \left(\frac{\delta\omega}{2} \right) \left(p_A - p_B - \tau \left(\frac{1}{T_{2A}} - \frac{1}{T_{2B}} \right) \right) \right] \quad (50)$$

where $\tau = \frac{\tau_A \tau_B}{\tau_A + \tau_B}$ (51)

Multiplying by the complex conjugate of the denominator D^* and retaining only the imaginary part v , the final expression for the intensity is obtained:

$$v(\omega) = \frac{\omega_1 M_0 (PS + QR)}{P^2 + R^2} \quad (52)$$

where $P = \frac{p_A}{T_{2A}} + \frac{p_B}{T_{2B}} + \tau \left(\frac{1}{T_{2A}T_{2B}} - \Delta\omega^2 + \left(\frac{\delta\omega}{2} \right)^2 \right)$

$$\begin{aligned}
 Q &= \tau \left(\Delta\omega - (p_A - p_B) \frac{\delta\omega}{2} \right) \\
 R &= \Delta\omega \left(1 + \tau \left(\frac{1}{T_{2A}} + \frac{1}{T_{2B}} \right) \right) \\
 &+ \frac{\delta\omega}{2} \left(p_A - p_B - \tau \left(\frac{1}{T_{2A}} - \frac{1}{T_{2B}} \right) \right)
 \end{aligned} \tag{53}$$

$$S = 1 + \tau \left(\frac{p_B}{T_{2A}} + \frac{p_A}{T_{2B}} \right)$$

This equation can be applied to the exchange between a quadrupole splitting type of spectrum and a single line. In order to calculate the effect of exchange on that kind of spectrum, the intensity of the quadrupole splitting resonances must be calculated. This was accomplished by defining a reduced frequency ξ (65):

$$\xi_{\pm} = \frac{\nu_{\pm} - (g\beta_N H_0/h)}{(3/4)(e^2 q Q/h) S_{CD}} = \pm \frac{3\cos^2\theta - 1}{2} \tag{54}$$

where $1 \geq \xi_+ \geq -1/2$ and $-1 \leq \xi_- \leq 1/2$ and the probability $p(\xi_{\pm})$ is:

$$p(\xi_{\pm}) = \frac{1}{\sqrt{\pm 2\xi_{\pm} + 1}} \quad (55)$$

Those probabilities are plotted in Fig. 12 and it is seen that when $-1/2 < \xi < 1/2$, $p(\xi)$ is the sum of $p(\xi_{+})$ and $p(\xi_{-})$ but when $1/2 \leq \xi \leq 1$ or $-1 \leq \xi \leq -1/2$, $p(\xi)$ corresponds to $p(\xi_{+})$ or $p(\xi_{-})$, respectively.

Dividing the quadrupole splitting pattern into its line components of angular frequency ω_A and transverse relaxation time T_{2A} , each line can be treated as a doublet in exchange with a central single resonance of angular frequency ω_B and transverse relaxation time T_{2B} . The resulting intensity is calculated using Eq. 52 which must be multiplied by $p(\omega_A)$. It is possible to vary the linewidth of the powder pattern components across the spectrum:

$$\Delta\nu_{1/2A} = \Delta\nu_{1/2A}^0 + \Delta\nu_{1/2A}' \frac{3\cos^2\theta - 1}{2} \quad (56)$$

where $\Delta\nu_{1/2A}^0$ and $\Delta\nu_{1/2A}'$ are the intrinsic and angle dependent linewidths of the quadrupole splitting components, respectively, from which the T_{2A} values can be calculated:

$$T_{2A} = \frac{1}{\pi\Delta\nu_{1/2A}} \quad (57)$$

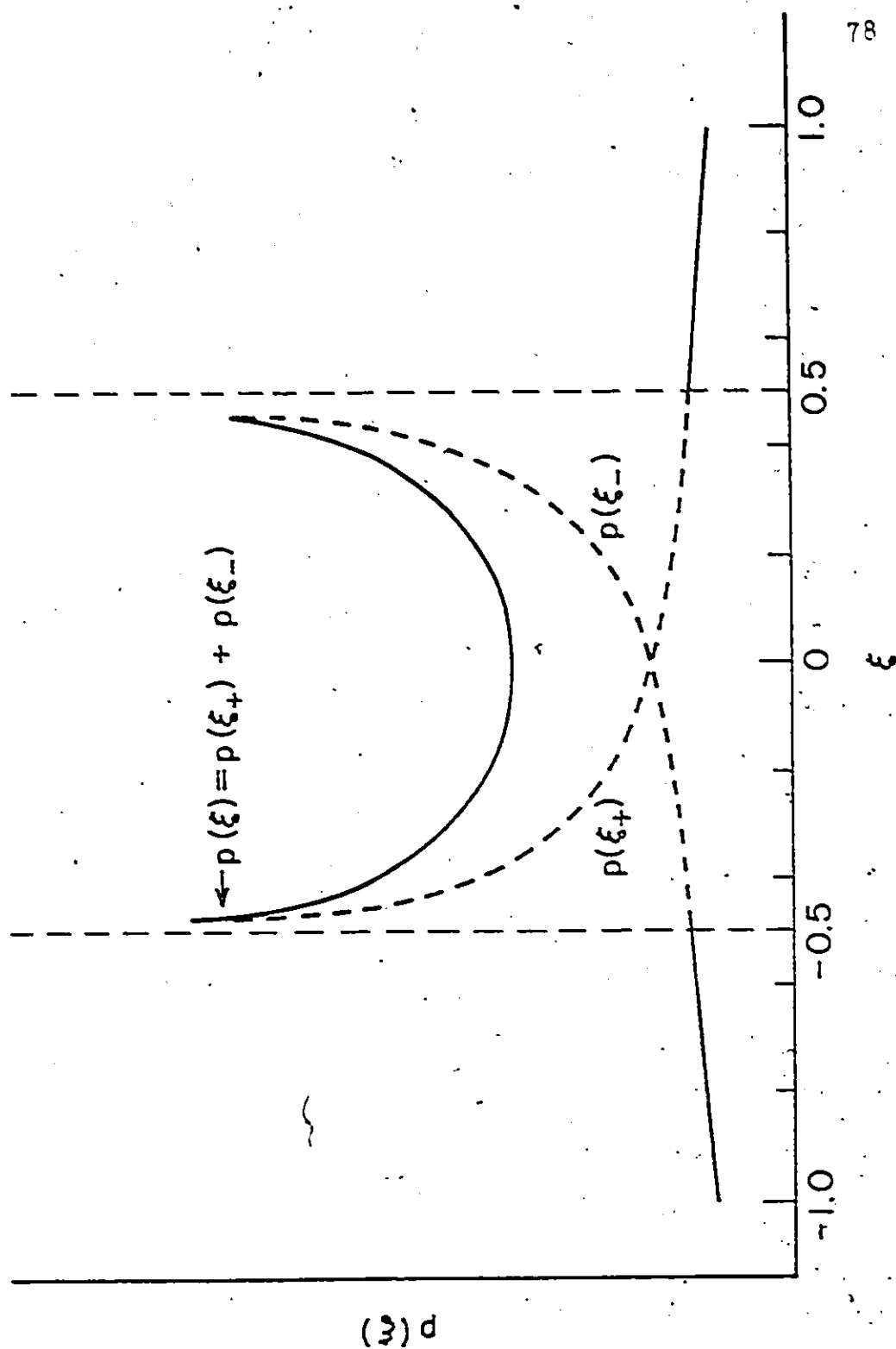


Fig. 12. Probability $p(\xi)$ as a function of reduced frequency ξ for a ^2H NMR quadrupole splitting.

A computer program has been written making use of these equations and is given in Table 3. Also included in the program is the calculation of ^2H NMR spectra in the absence of exchange (65).

TABLE 3

Fortran program to simulate ^2H NMR spectra in
absence and/or presence of chemical exchange

```

0000100  C  QUADEXCH PROGRAM TO CALCULATE DEUTERIUM NMR SPECTRA
0000200  C  IN THE ABSENCE OF EXCHANGE AND/OR IN THE PRESENCE
0000300  C  OF EXCHANGE BETWEEN A QUADRUPOLE SPLITTING AND A
0000400  C  SINGLE LINE WITH POSSIBLE ADDITION OF SPECTRA
0000500  C  QUADRUPOLE SPLITTING LINESHAPE CALCULATED ACCORDING TO
0000600  C  SEELIG, QUART. REV. BIOPHYS., 1977, P353 USING
0000700  C  A LORENTZIAN LINESHAPE FOR EACH COMPONENT
0000800  C  EFFECT OF EXCHANGE CALCULATED USING THE THEORY
0000900  C  OF GUTOVSKY AND HOLM, J. CHEM. PHYS., VOL 5, 1956, P1228
0001000  C  MODIFIED FOR VARIABLE LINEWIDTH
0001100  C  INPUT PARAMETERS
0001200  C  NQUAD = NUMBER OF QUADRUPOLE SPLITTINGS NOT IN EXCHANGE
0001300  C  NEXCH = NUMBER OF QUADRUPOLE SPLITTINGS IN EXCHANGE WITH
0001400  C  A SINGLE LINE
0001500  C  XNP = NUMBER OF POINTS
0001600  C  SW = SWEEP WIDTH IN HZ
0001700  C  QCC = QUADRUPOLE COUPLING CONSTANT IN HZ
0001800  C  QQ = QUADRUPOLE SPLITTING IN HZ
0001900  C  SIGMA1 = INTRINSIC LINEWIDTH IN HZ
0002000  C  SIGMA2 = ANGLE DEPENDENT LINEWIDTH IN HZ
0002100  C  PROB(NQ) = PROBABILITY OF THE QUADRUPOLE SPLITTING NQ
0002200  C  TEXCHQ = RESIDENCE TIME OF THE QUADRUPOLE SPLITTING IN SEC
0002300  C  TEXCHL = RESIDENCE TIME OF THE SINGLE LINE IN SEC
0002400  C  XLWQ = INTRINSIC LINEWIDTH OF THE QUADRUPOLE SPLITTING IN HZ
0002500  C  XLWW = ANGLE DEPENDENT LINEWIDTH OF THE QUADRUPOLE
0002600  C  SPLITTING IN HZ
0002700  C  XLWL = LINEWIDTH OF THE SINGLE LINE IN HZ
0002800  C  PROB(NT) = TOTAL PROBABILITY OF THE QUADRUPOLE SPLITTING
0002900  C  AND LINE NT IN EXCHANGE
0003000  C  FIELD2 = ANGULAR FREQUENCY OF THE SINGLE LINE IN SEC -1
0003100  C  OUTPUT PARAMETERS
0003200  C  SPECT(I) = INTENSITY AT POINT I
0003300  C  FREQ(I) = FREQUENCY AT POINT I IN HZ
0003400  C  SPECTRUM CENTERED AT FREQ(1) = 0.0
0003500  C  DIMENSION P(1001),FIELD(1001),DFIELD(1001),SFIELD(1001)
0003600  C  DIMENSION P(1001),Q(1001),R(1001),XNU(1001),E(1001),T2Q1(1001)
0003700  C  DIMENSION EINT(1001),EXINT(10,1001),FREQ(1001),SE(1001)
0003800  C  DIMENSION AREA(10),PROB(10),SPECT(1001),SIGMA(1001),S(1001)
0003900  C  DATA FIELD2/0.0/
0004000  C  READ(6,11)NQUAD
0004100  C  READ(6,11)NEXCH
0004200  C  READ(6,10)XNP
0004300  C  READ(6,10)SW
0004400  C  READ(6,10)QCC
0004500  C  NP=XNP
0004600  C  10 FORMAT(F16.9)
0004700  C  11 FORMAT(I2)
0004800  C  K=NP/2+1
0004900  C  PI=2.*ARCOS(0)
0005000  C  PROBT=0.0
0005100  C  -NTOT=NQUAD+NEXCH
0005200  C  NP=NP+1
0005300  C  ST=-SW/(1.5*QCC)
0005400  C  DIF=2.*ST/XNP

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0005400 DO 140 I=1,K
0005500 E(I)=ST
0005600 FREQ(I)=E(I)*0.75*QCC
0005700 J=NP-I+1
0005800 E(J)=-E(I)
0005900 FREQ(J)=-FREQ(I)
0006000 SPECT(I)=0.0
0006100 SPECT(J)=0.0
0006200 ST=ST-DIF
0006300 140 CONTINUE
0006400 IF(NQUAD.EQ.0)GO TO 110
0006500 DO 100 NQ=1,NQUAD
0006600 READ(6,10)DQ
0006700 READ(6,10)SIGMA1
0006800 READ(6,10)SIGMA2
0006900 READ(6,10)PROB(NQ)
0007000 ER=DQ/(0.75*QCC)
0007100 SIGMA1=SIGMA1/(0.75*QCC*ER)
0007200 SIGMA2=SIGMA2/(0.75*QCC*ER)
0007300 AREA(NQ)=0.0
0007400 DO 20 I=1,K
0007500 VAL=E(I)*2./ER
0007600 IF(E(I).LE.-ER)GO TO I2
0007700 IF(E(I).LE.-ER/2.)GO TO I4
0007800 PE(I)=1./SQRT(VAL+1.)+1./SQRT(-VAL+1.)
0007900 GO TO I8
0008000 14 PE(I)=1./SQRT(ABS(VAL+1.))
0008100 GO TO I8
0008200 12 PE(I)=0.0
0008300 18 SIGMA(I)=SIGMA1-SIGMA2*E(I)/ER
0008400 EXINT(NQ,I)=0.0
0008500 J=NP-I+1
0008600 PE(J)=PE(I)
0008700 SIGMA(J)=SIGMA(I)
0008800 EXINT(NQ,J)=0.0
0008900 20 CONTINUE
0009000 DO 30 I=1,K
0009100 DO 32 J=1,NP
0009200 SE(I)=PE(J)/(PI*SIGMA(J)*((1.+4.*((E(I)-E(J))/ER)**2/SIGMA(J)**2))
0009300 EXINT(NQ,I)=EXINT(NQ,I)+SE(I)
0009400 AREA(NQ)=AREA(NQ)+SE(I)
0009500 32 CONTINUE
0009600 JR=NP-I+1
0009700 EXINT(NQ,JR)=EXINT(NQ,I)
0009800 30 CONTINUE
0009900 AREA(NQ)=2.*AREA(NQ)-EXINT(NQ,K)
0010000 100 CONTINUE
0010100 IF(NEXCH.EQ.0)GO TO 120
0010200 110 DO 142 I=1,K
0010300 FIELD(I)=FREQ(I)*2.*PI
0010400 DFIELD(I)=(FIELD(I)-FIELD2)*2.
0010500 SFIELD(I)=(FIELD(I)+FIELD2)/2.
0010600 J=NP-I+1
0010700 FIELD(J)=-FIELD(I)
0010800 142 CONTINUE
0010900 DO 130 NE=1,NEXCH
0011000 NT=NQUAD*NE
0011100 READ(6,10)DQ
0011200 READ(6,10)TEXCHQ
0011300 READ(6,10)TEXCHL
0011400 READ(6,10)XL*Q
0011500 READ(6,10)XL*VQ
0011600 READ(6,10)XL*L
0011700 READ(6,10)PROB(NT)
0011800 STEXCH=TEXCHQ*TEXCHL
0011900 TAU=TEXCHQ*TEXCHL/STEXCH

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0012000 PQ=TEXCHQ/STEXCH
0012100 PL=TEXCHL/STEXCH
0012200 T2LI=2.*PI*XLWL
0012300 DP=PQ-PL
0012400 ER=DP/(0.75*QCC)
0012500 TOTAL=0.0
0012600 AREA(NT)=0.0
0012700 DO 21 I=1,K
0012800 VAL=E(I)*2./ER
0012900 IF(E(I).LE.-ER)GO TO 13
0013000 IF(E(I).LE.-ER/2.)GO TO 15
0013100 PE(I)=1./SQRT(VAL+1.)*1./SQRT(-VAL+1.)
0013200 GO TO 19
0013300 15 PE(I)=1./SQRT(ABS(VAL+1.))
0013400 GO TO 19
0013500 13 PE(I)=0.0
0013600 19 TOTAL=TOTAL+PE(I)
0013700 T2QI(I)=2.*PI*(XLWQ-XLWVQ*E(I)/ER)
0013800 S(I)=1.+TAU*(PL*T2QI(I)+PQ*T2LI)
0013900 EXINT(NT,I)=0.0
0014000 21 CONTINUE
0014100 DO 44 KE=1,K
0014200 DO 42 I=1,NP
0014300 P(I)=TAU*(T2QI(KE)*T2LI-(SFIELD(KE)-FIELD(I))*2+DFIELD(KE)*2)+PQ-
0014320 *T2QI(KE)+PL*T2LI
0014400 Q(I)=TAU*(SFIELD(KE)-FIELD(I)-OP*DFIELD(KE))
0014500 R(I)=(SFIELD(KE)-FIELD(I))*(1.+TAU*(T2QI(KE)+T2LI))+DFIELD(KE)*(OP-
0014520 *TAU*(T2LI-T2QI(KE)))
0014600 XNU(I)=((1.+TAU*T2INV)*P(I)+Q(I)*R(I))/(P(I)*2+R(I)*2)
0014700 EINT(I)=XNU(I)*PE(KE)/TOTAL
0014800 EXINT(NT,I)=EXINT(NT,I)+EINT(I)
0014900 42 CONTINUE
0015000 44 CONTINUE
0015100 DO 70 I=1,K
0015200 L=NP-I+1
0015300 EXINT(NT,I)=EXINT(NT,I)+EXINT(NT,L)-EINT(I)
0015400 70 CONTINUE
0015500 M=K-1
0015600 DO 50 J=1,M
0015700 I=K+J
0015800 L=K-J
0015900 EXINT(NT,I)=EXINT(NT,L)
0016000 AREA(NT)=AREA(NT)+EXINT(NT,I)
0016100 50 CONTINUE
0016200 AREA(NT)=2.*AREA(NT)+EXINT(NT,K)
0016300 CONTINUE
0016400 130 DO 144 I=1,NTOT
0016500 PROBT=PROBT+PROB(I)
0016600 144 CONTINUE
0016700 DO 147 I=1,NP
0016800 DO 145 J=1,NTOT
0016900 SPECT(I)=SPECT(I)+PROB(J)*EXINT(J,I)/(PROBT*AREA(J))
0017000 145 CONTINUE
0017100 147 CONTINUE
0017200 WRITE(8,17) (SPECT(I),FREQ(I),I=1,NP)
0017300 17 FORMAT(6E13.6)
0017400 50 STOP
0017500 END

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III. EXPERIMENTAL

A. Materials

Procaine hydrochloride (PRC) and tetracaine hydrochloride (TTC) were purchased from Sigma Chemical Company, Saint-Louis, USA. Phosphatidylethanolamine (PE, egg yolk), phosphatidylcholine (PC, egg yolk), phosphatidylserine (PS, beef brain) and phosphatidylinositol (PI, wheat germ) were obtained from Lipid Products, Surrey, England. PC was also extracted from eggs (82) or obtained from Pfanstiehl Laboratories, Waukegan, USA in an unpurified form. PC-5-SL was a generous gift of P. Laks, Simon Fraser University, Burnaby, Canada. 1,2-dipalmitoyl- d_{62} -sn-glycero-3-phosphocholine (DPPC- d_{62}) was purchased from Lipid Specialties Company, Boston, USA. Phosphatidylethanolamine-2,2,3,3- d_4 (PE- d_4) was a generous gift of M. G. Taylor, National Research Council, Ottawa, Canada. Palmitic acid-2,2- d_2 , palmitic acid-6,6- d_2 , palmitic acid-12,12- d_2 and palmitic acid-16,16,16- d_3 were synthesized according to a published procedure by Dr. A. P. Tulloch, National Research Council, Saskatoon, Canada (83). Palmitic acid- d_{31} was obtained from Larodan Fine

Chemicals, Malmö, Sweden. 1-palmitoyl-lysophosphatidylcholine was obtained from Serdary Research Laboratories, London, Canada. Iodomethane- d_3 , dimethylamine- d_6 and deuterium oxide (99.7% d) were purchased from Merck. Sharp and Dohme, Pointe-Claire, Canada. p-Nitrobenzoylchloride, dimethylaminoethanol and hexadecyltrimethylammonium hydroxide were obtained from Eastman, Rochester, USA. Dicyclohexylcarbodiimide, N,N-dimethylaminopyridine and deuterium depleted water were purchased from Aldrich Chemical Company, Milwaukee, USA. Silicic acid (Bio-Sil) was from Bio-Rad Laboratories, Richmond, USA. Ion exchange resin (Rexyn 102) was obtained from Fisher, Pittsburgh, USA. Thin layer chromatography was performed on precoated Merck silica gel plates. All solvents used for chromatography were distilled prior to use. All other chemicals were analytical reagent grade.

B. Syntheses

(a) Procaine-d₂ and Tetracaine-d₂

Procaine and tetracaine (5 g) were refluxed in D₂O (100 ml) with 3-4 drops of DCl for periods ranging from 48 to 96 hours for the former, and of 24 hours for the latter. The reaction was followed by ¹H NMR until most of the signal due to the aromatic protons ortho to the nitrogen had disappeared. Water was evaporated under vacuum and the residue recrystallized from absolute ethanol: m.p., PRC-d₂.HCl 157°C, TTC-d₂.HCl 146-149°C; analysis: TTC-d₂.HCl calcd., C 59.49, H 8.38, N 9.25, Cl 11.70, found, C 58.99, H 8.48, N 9.12, Cl 11.88; PRC-d₂.HCl calcd., C 57.03, H 8.10, N 10.23, Cl 12.95; found, C 56.61, H 7.93, N 10.09, Cl 12.72; both compounds showed greater than 90% d from ¹³C NMR.

(b) Procaine-d₄

Diacetamide was first prepared by adding acetyl chloride (78.5 g; 1.0 mole) to acetamide (60 g; 1.0 mole) in acetic anhydride (100 g; 1.0 mole), while stirring and heating under a reflux condenser. A white solid separated slowly from the solution and hydrogen chloride was evolved. After three hours, the reaction

mixture was cooled slightly and benzene (300 ml) was added. The yellow hygroscopic solid was removed by filtration. Benzene was removed by distillation on a steam bath and acetic acid by pumping on a vacuum line, to yield a crystalline product. The crude diacetamide, (60 g; m.p. 77-78°C) was purified by vacuum sublimation under 0.02 mm Hg from a bath heated to 65°C. The hard white crystals melted at 80-81°C.

The diacetamide was reduced to the labelled amine with lithium aluminum deuteride in ether. Twenty-five grams (0.25 mole) of sublimed diacetamide in ether were added dropwise (100 ml) to a suspension of lithium aluminum deuteride (10.0 g) in 450 ml of absolute ether and the reaction mixture was stirred for 10 hours. The complex salt and excess deuteride were decomposed by careful addition of 3 N hydrochloric acid until the reaction mixture was acidic. The ether was then evaporated, and the residue made alkaline by addition of 50% sodium hydroxide before distillation with steam. The distillate was collected in 20 ml concentrated hydrochloric acid diluted with 60 ml of water. The distillate was freed of water and excess acid in the rotary evaporator under reduced pressure. The residue was dissolved in 300 ml chloroform and distilled for azeo-

tropic elimination of the residual solvent. Suspended ammonium chloride was removed by filtration and the residual chloroform evaporated. The yield of light brown diethylamine-1,1,1',1'-d₄ hydrochloride was 22.5 g.

The free base was obtained by adding 50% sodium hydroxide to the salt in an evacuated system and condensing the liberated base in a trap cooled in dry ice. The diethylamine-1,1,1',1'-d₄ was dried over a few pellets of potassium hydroxide and redistilled into another trap. The yield was 10.0 ml (mass analysis: 85% d₄, 6.5% d₃, minor amounts of d₂ and d₁).

Diethylamine-1,1,1',1'-d₄ (10 ml, 0.10 mole) was reacted for one hour with an equimolar amount (5 ml, 0.10 mole) of ethylene oxide containing one drop of water in a round bottom flask at dry ice temperature (84). The flask was then heated in a water bath (50-60 °C) for 20 hours and finally the diethyl-1,1,1',1'-d₄-aminoethanol was distilled on a vacuum line.

Diethyl-1,1,1',1'-d₄-aminoethanol (7 g, 0.06 mole) was dissolved in dry benzene (100 ml) to which were added p-nitrobenzoylchloride crystals (15 g, 0.08 mole) followed by reflux for one hour. After cooling, the crystals were filtered and washed with benzene.

The resulting 2-diethyl-1,1,1',1'-d₄-aminoethyl-

4-nitrobenzoate was dissolved in methanol, the solution placed in a reducing apparatus with 0.2 g platinum oxide and shaken under 25 p.s.i. of hydrogen for ca 15 hours. The platinum was removed by filtration, the methanol evaporated under reduced pressure, and procaine- d_4 recrystallized from absolute ethanol (m.p. 153-5°C, 85% d from 1H NMR; analysis: C 56.89, H 8.02, N 10.36, Cl 12.72).

(c) Tetracaine- d_3

A solution of 3-chloropropene (60 g, 0.6 mole) and dibenzoylperoxide (2.0 g) in bromotrichloromethane (300 ml) was heated under reflux for six hours. Excess bromotrichloromethane was then removed under reduced pressure (6.0 mm Hg) in a rotary evaporator. The residue was fractionated in a Vigreux column with partial take-off stillhead under 0.1-0.2 mm Hg and collected in a receiver cooled in ice-water. The yield of 3-bromo-1,1,1,4-tetrachlorobutane was 188 g (b.p. 46°C/0.1 mm Hg).

Acetic anhydride (100 ml) was shaken in a separatory funnel with deuterium oxide (10.0 ml) to remove traces of acetic acid. It was then stirred in a 250 ml round bottom flask with deuterium oxide (21 ml) to

which a drop of acetyl chloride had been added. The solution was slowly heated to boiling to generate acetic acid- d_1 .

In a 500 ml round bottom flask fitted with a thermometer, a separatory funnel, and reflux condenser to which a spiral trap and a drying tube were connected, were placed zinc dust (50.0 g) and acetic acid- d_1 (75 ml). The spiral trap was immersed in a Dewar cooled with dry ice. 1,1,1,4-Tetrachloro-3-bromobutane (60.0 g, 0.22 mole) was added dropwise to the stirred zinc dust and acetic acid- d_1 . An exothermic reaction occurred with evolution of deuterated butene. The rate of addition of halide was adjusted to keep the reaction mixture at 80-90°C. When the addition of halide was completed, the apparatus was swept with a current of dry nitrogen or argon. The yield of liquid 1-butene-4,4,4- d_3 in the trap was 10.8 g.

Dichloromethane (50 ml) and dibenzoyl peroxide (2.0 g) were placed in a 300 ml glass tube and attached to a vacuum line through a U-shaped trap. The contents of the tube were frozen in liquid nitrogen and evacuated. 1-Butene-4,4,4- d_3 (25.0 ml) was distilled into the tube from the trap. The tube was allowed to come to -10-0°C and addition of hydrogen bromide from a cy-

linder was started while stirring and cooling in ice-water. The pressure in the system was carefully monitored with a manometer. After three hours, no more hydrogen bromide was absorbed and the reaction mixture turned pale yellow (free bromine).

The reaction mixture was stirred for one hour, washed with water, dilute sodium bisulfite, and water, and dried over anhydrous potassium carbonate. The solvent was distilled in a suitable column (spinning band or concentric tube type). After collecting 1.0-1.5 ml of a mixture of n- and iso-butane, pure 1-bromobutane-4,4,4-d₃ was obtained at 99-100°C/760 mm Hg (yield, 26.5 g).

p-Aminobenzoic acid (8 g, 0.06 mole) was neutralized with potassium bicarbonate in water until dissolution was complete. 1-Bromobutane-4,4,4-d₃ (7 g, 0.05 mole) was added and the solution refluxed for about 5 hours. Two products are obtained, N-butyl-4,4,4-d₃-p-aminobenzoic acid and N,N-dibutyl-4,4,4,4',4',4'-d₆-p-aminobenzoic acid. The solution was made acidic and the precipitate was filtered and washed with methylene chloride to remove the N,N-dibutyl-4,4,4,4',4',4'-d₆-p-aminobenzoic acid.

The residue (7.3 g) which contained unreacted

p-aminobenzoic acid and N-butyl-4,4,4-d₃-p-aminobenzoic acid was put in a reflux apparatus with an excess of ethanol (100 ml). The solution was saturated with HCl gas (2 hours) and refluxed overnight under a slow stream of HCl. Ethanol was evaporated and the residue dissolved in water. Sodium acetate was added to neutralize the solution and to precipitate ethyl N-butyl-4,4,4-d₃-p-aminobenzoate. When the first traces of red appeared (due to ethyl p-aminobenzoate), the solution was filtered and the product dried at room temperature.

The ethyl N-butyl-4,4,4-d₃-p-aminobenzoate was transesterified with an excess of dimethylaminoethanol (100 ml) and sodium ethoxide as catalyst for 10 hours. Ethanol was evaporated under reduced pressure and the excess dimethylaminoethanol was removed on a vacuum line. The residue was dissolved in ether and extracted with 20 ml fractions of an aqueous solution of 0.1 N HCl. When the pH became acidic, the extracts were combined, the water evaporated, and the tetracaine-d₃ recrystallized from ethanol (m.p. 147-148°C; analysis: C 59.04, H 8.41, N 9.33, Cl 11.52).

(d) Tetracaine-d₆

Dimethylamine-d₆ (5 g, 0.10 mole) was reacted

with ethylene oxide (4.9 ml, 0.10 mole) in the same manner as diethylamine-1,1,1',1'-d₄ (see procaine-d₄) to yield 3.5 g of dimethyl-d₆-aminoethanol (b.p. 134°C).

Dimethyl-d₆-aminoethanol (3.5 g, 0.037 mole) was dissolved in dry benzene and p-nitrobenzoyl chloride added (10 g, 0.54 mole). The mixture was refluxed for one hour, cooled, filtered and washed with benzene. The product was dried and dissolved in water. The aqueous solution was filtered to remove p-nitrobenzoic acid, made alkaline with potassium bicarbonate, and extracted with ether. The ether solution was dried over potassium carbonate and the solvent evaporated.

The 2-dimethyl-d₆-aminoethyl p-nitrobenzoate (6.3 g, 0.026 mole) was dissolved in ethanol with 0.3 g of platinum oxide and the hydrogenation was carried out (25 p.s.i. ca 16 hours). After a decrease of 7 p.s.i. in pressure, butyraldehyde (5.6 g, 0.078 mole) was added and the reduction continued for 24 hours. The residue was dissolved in ether, extracted with aqueous 0.1 N HCl solution and the resultant tetracaine-d₆ was recrystallized from ethanol (m.p. 147-148°C; analysis: C 58.24 H 8.40, N 9.14, Cl 11.17).

(e) Phosphatidylcholine- d_9

Egg phosphatidylethanolamine (0.4 g) was mixed with methyl- d_3 iodide (1 g) in the presence of potassium bicarbonate (2 g) in chloroform: methanol (1:1) (S5, S6), and the solution saturated with nitrogen (10 min). The mixture was stirred at 40°C until all the PE had disappeared, as checked by TLC using chloroform: methanol: water 65: 25: 4 (rf: PC 0.3, PE 0.6). When the conversion to phosphatidylcholine- d_9 was completed, the potassium bicarbonate was removed by filtration and most of the solvent was evaporated. Chloroform was added and the solution filtered again to remove potassium iodide. The residual solution was chromatographed on a silicic acid column (15 g) eluted with methanol/chloroform (2-40% methanol). PC- d_9 was eluted at 40% methanol as indicated by TLC; PC- d_9 was then isolated and stored in methanol: chloroform 1:1 at -20°C (no $(CH_3)_3N$ signal by 1H NMR).

(f) Phosphatidylcholine-3,3,4,4- d_4

PE- d_4 was obtained by the action of phospholipase D and deuterated ethanolamine on egg PC. PE- d_4 was then converted to PC- d_4 by reaction with iodomethane in presence of potassium bicarbonate following the PC- d_9

synthesis procedure (see Section e).

(g) 1-Palmitoyl,2-deuteropalmitoyl-sn-glycero-3-phosphocholine

Selectively deuterated palmitic acid (1.27 g, 5 mmole) was dissolved in dry carbon tetrachloride (75 ml) and the solution added to a dicyclohexycarbodiimide (0.51 g, 2.5 mmole) solution in dry carbon tetrachloride (25 ml) (87). After 12 hours at room temperature, the precipitated dicyclohexyl urea was removed by filtration and the solvent evaporated to yield white crystals of deuterated palmitic anhydride (IR bands at 1750 and 1810 cm^{-1} : carboxylic anhydride).

1-Palmitoyl lysophosphatidylcholine (50 mg, 0.1 mmole), deuterated palmitic anhydride (270 mg, 0.39 mmole) and finely powdered sodium oxide (3 mg) were mixed in a dry flask under vacuum (88,89). While stirring, the mixture was heated at 60°C. Periodically, the reaction mixture was monitored by TLC (chloroform: methanol: water 65:25:4). When the reaction was completed (12 hours), the reaction mixture was dissolved in chloroform, filtered, applied to a silicic acid chromatography column and eluted with methanol: chloroform (gradient 2-40% methanol). The fractions contain

ning deuterated DPPC, identified by TLC, were collected and evaporated (yield 50%).

(h) 1,2-Dipalmitoyl- d_{62} -sn-glycero-3-phosphocholine

Crude egg yolk PC (30 g) was dissolved in diethyl ether (300 ml) and a 25% solution of hexadecyltrimethylammonium hydroxide in methanol (30 ml) was added (90). The solution was stirred for 30 min. and then allowed to settle. The supernatant solution was discarded and the precipitate residue rinsed with two 20 ml portions of ether. The white solid residue was dissolved in water (15 ml at 70°C) and the pH of the solution was adjusted at 4.0 with concentrated HCl. The solution was treated with charcoal. Cadmium chloride (5.0 g) was dissolved in water (10 ml) and the solution was added along with 95% ethanol (300 ml). The residual milky solution was cooled and the glycerophosphocholine cadmium chloride complex precipitated (yield 40%, TLC in methanol: water 7: 3 rf 0.5).

The glycerophosphocholine cadmium chloride complex (100 mg, 0.24 mmole) was rendered anhydrous by repeated evaporation of added dry benzene (91). The solid residue was suspended in freshly distilled dry chloroform (6ml distilled over P_2O_5). Palmitic anhy-

dride- d_{62} (see Section g)(341 mg, 0.6 mmole) and N,N-dimethyl-4-aminopyridine (60 mg, 0.48 mmole) were added and the flask was sealed under dry nitrogen. The reaction mixture, protected from light, was stirred at room temperature for about 30 hours. The solvent was evaporated and the residue treated with methanol: chloroform: water 5: 4: 1 (10 ml). The insoluble solid residue was removed by filtration and the filtrate passed through an acidic resin column (Rexyn 102). The column was washed with two bed volumes of the same solvent and the solution was evaporated and lyophilized to remove water. The residue was dissolved in chloroform and applied to a silicic acid chromatography column (15 g) eluted with methanol: chloroform (gradient 2-40%). The fractions containing DPPC- d_{62} , identified by TLC, were collected and evaporated (r.f. = 0.3 in $CHCl_3$: MeOH: H_2O 65: 25: 4).

C. Methods

(a) Preparation of samples

Lipid solutions in chloroform:methanol were evaporated under nitrogen and placed under vacuum overnight to remove the residual solvent. When deuterated anesthetics were used, the dry lipid and local anesthetic were dispersed in buffer by shaking with a vortex mixer (5-10 min.). For the ESR experiments, or when deuterated lipid was used, spectra were first run in the absence of anesthetic. Thus, in these cases, the latter was added to already dispersed phospholipid.

Two buffers were used, a phosphate buffer with a pH range between 4.5-8.5 and a borate-phosphate-citrate buffer (BPC) with a pH range between 2.0-12.0 (92). For ^2H NMR samples, these buffers were usually prepared in deuterium depleted water to eliminate the peak due to deuterium at natural abundance in water.

The phosphate buffer was prepared by mixing two solutions: (1) 0.025 N sodium phosphate dibasic $\text{NaHPO}_4 \cdot 7\text{H}_2\text{O}$ (1.675 g/500 ml), 0.1 N sodium chloride (2.925 g/500 ml) and (2) 0.025 N sodium phosphate monobasic $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (1.725 g/500 ml). The suitable pH was obtained by mixing solution (1) (pH 8.5) and solution (2)

(pH 4.5) in the required proportions.

The BPC buffer was prepared by dissolving citric acid (0.135 g), 85% phosphoric acid (0.062 ml), boric acid (0.071 g) and sodium hydroxide (0.274 g) in water (50 ml) in a 100 ml volumetric flask. The pH was adjusted with concentrated HCl (0.181 ml at pH 5.5; 0.069 ml at pH 9.5) and the volume was completed with water. The final concentrations were 0.02 N sodium citrate, 0.02 N sodium phosphate, 0.017 N sodium borate and 0.1 N sodium chloride.

The sample pH values were measured and corrected if necessary. Samples were maintained under nitrogen at all times to prevent lipid oxidation (if unsaturated).

(b) Determination of pK_a values

Local anesthetics PRC and TTC contain two titratable amino groups: an aromatic primary (PRC) or secondary (TTC) and a tertiary amino groups (Fig. 5). The pK_a values of the former are low and were determined by ^{13}C NMR whereas the pK_a values of the latter are high and were determined by electrometric titration. The pK_a value of the tertiary amino group cannot be determined by ^{13}C NMR because of the low solubility of the local anesthetics at high pH values (see Section IV.B.d).

The ^{13}C NMR spectra of local anesthetics (0.5 M in D_2O) were recorded at several pH values between 1.0 and 7.5. The chemical shifts of the carbon atoms adjacent to the aromatic amino group were sensitive to pH, allowing determination of the pK_a values.

In order to determine the pK_a values of the tertiary amino group, PRC or TTC (26 mM) were dissolved in water at low pH (about 2) and titrated slowly with 0.01 N NaOH, until pH 11, as monitored by a pH meter. Experiments were also performed at different local anesthetic concentrations (1-50 mM) and in the presence of PC dispersions (52 mM).

(c) Determination of partition coefficients (K_p)

Dispersions of lipids PC, PC-PS or PC-PI (13.5 mM) in BPC buffer (2ml) containing PRC or TTC (6.8 mM) were centrifuged at 20,000 r.p.m. for 30 min. in order to separate the lipids from the anesthetic solution (59,93). An aliquot of the supernatant solution (1 ml) was taken and diluted with buffer at the same pH for spectrophotometric measurement of the anesthetic concentration (PRC, $\lambda_{\text{max}} = 307 \text{ nm}$; TTC, $\lambda_{\text{max}} = 286 \text{ nm}$)(Fig. 13). The ratio of the absorbance of the anesthetic in the sample to that of the initial solution was proportional to the

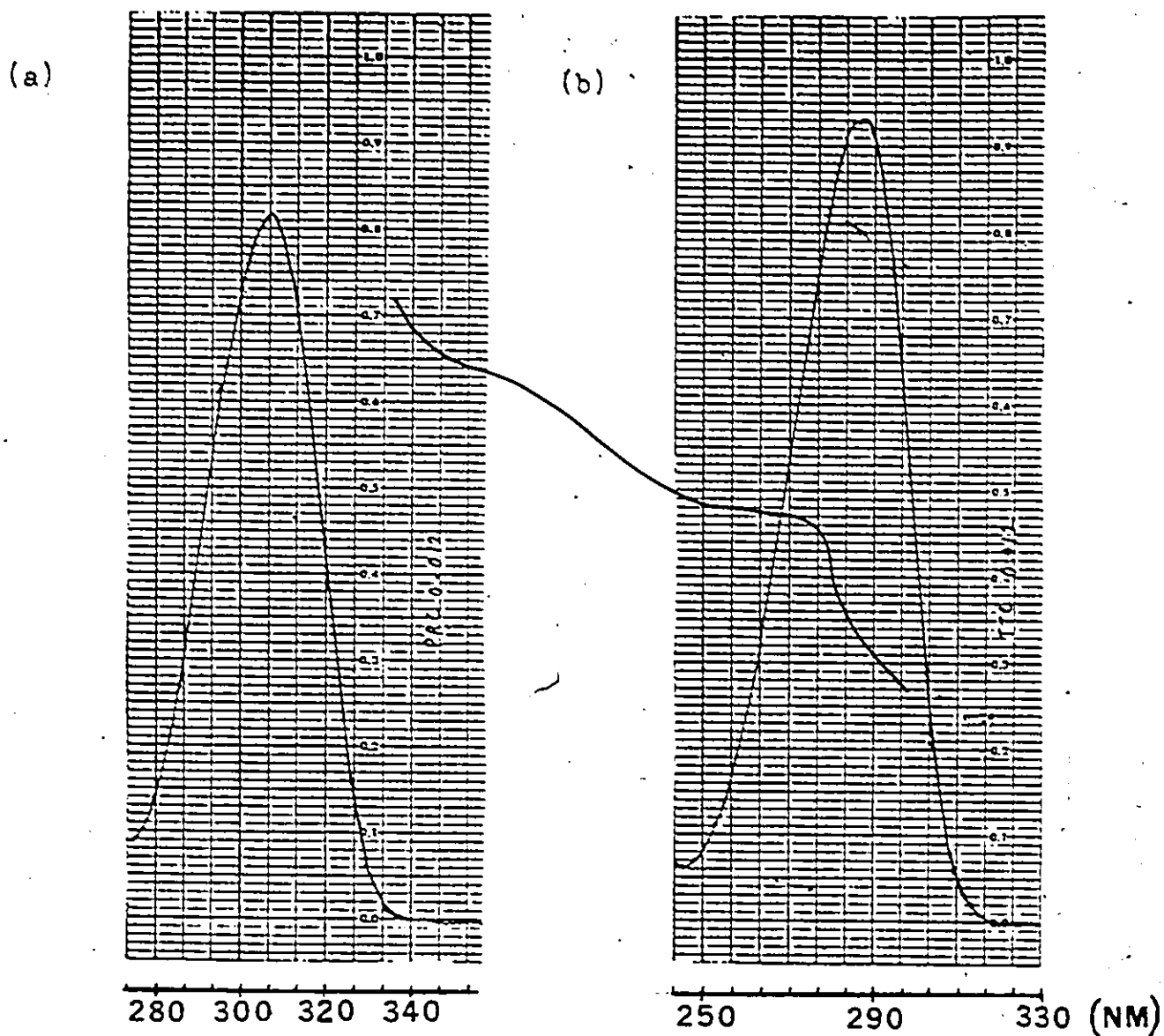


Fig. 13. UV-VIS spectra of (a) PRC ($\lambda_{\text{max}} = 307 \text{ nm}$) and (b) TTC ($\lambda_{\text{max}} = 286 \text{ nm}$). The spectra were taken with 0.012 mg/ml PRC (0.044 mM) and 0.012 mg/ml TTC (0.040 mM). The absorption coefficients are $18,700$ for PRC and $23,200$ for TTC ($\text{in cm}^{-1} \text{ M}^{-1}$).

amount of anesthetic in water. The amount bound to the lipid was calculated by difference. TLC (chloroform: methanol: water 65: 25: 4) of the supernate indicated no trace of lipid. Partition coefficients were measured at various ratios of local anesthetic to lipid (5-50 mole%) and lipid to water (1.35-13.5 mM).

(d) Determination of the solubility of local anesthetics in buffer at different pH values

Local anesthetic (PRC or TTC) was added to a BPC buffer solution until saturation. The pH was adjusted with concentrated NaOH or HCl solutions. The saturated anesthetic solution was centrifuged for 40 min. at 30°C and 15,000 r.p.m.. An aliquot of the supernatant (50 μ l) was taken and diluted with BPC buffer at pH 5.5 in order to determine its concentration by UV-VIS spectroscopy (PRC, $\lambda_{\text{max}} = 307$ nm; TTC, $\lambda_{\text{max}} = 286$ nm).

(e) Analysis of fatty acid composition of phospholipids

To dry phospholipid (5 mg) were added solutions of 0.4 mg/ml standard heptadecanoic acid (0.2 ml), 0.7 N methanolic HCl (4.5 ml) and a few boiling chips. The solution was refluxed for 1-2 hours and then cooled. Water (0.5 ml) was added and the solution was extracted

with petroleum ether (1-2 ml). The solvent was evaporated, the residue (fatty acid methyl esters) was dissolved in petroleum ether and identified on a gas chromatograph (Fig. 14).

(f) Study of local anesthetic and DPPC hydrolysis

The extent of local anesthetic hydrolysis was monitored by recording ^2H NMR spectra of a deuterated local anesthetic (26 mM) in PC dispersion (52 mM) at pH 9.5 in BPC buffer every 6 hours for a period of 36 hours at 30°C.

The hydrolysis of DPPC was studied by TLC (chloroform: methanol: water 65: 25: 4) of a DPPC dispersion (26 mM) in BPC buffer at several pH values (7.5, 8.0, 8.5, 9.0, 9.5). TLC were run at variable intervals for a period of 10 hours at 47°C (PC, rf 0.3; lyso PC, rf 0.2).

(g) Examination of variations in the phospholipid and local anesthetic particle size

In order to detect any change in the size of multilamellar lipid dispersions upon anesthetic addition or pH change, UV-VIS (200-450 nm) spectra of PC-PS dispersions (1.3-52 mM) in BPC buffer were recorded at pH

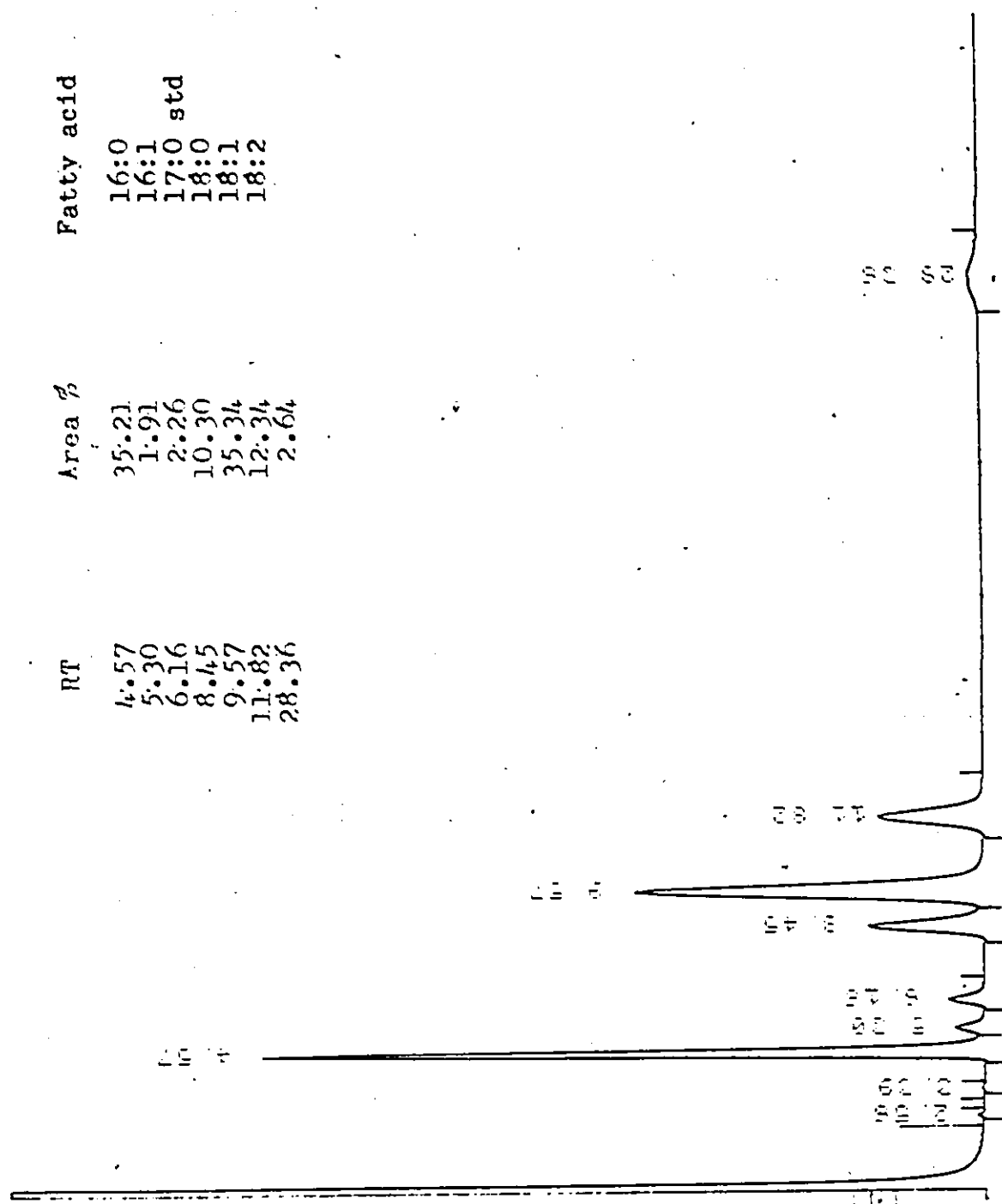


Fig. 14. Gas chromatograph spectrum of fatty acid methyl esters from egg PC (Lipid Products). The spectrum was obtained at 175°C on a DEGS column. Heptadecanoic acid was used as internal standard.

5.5 and 9.5, both in absence and presence of TTC (0.65-26 mM). An increase in light scattering is indicative of a reduction in particle size.

D. Equipment

^2H NMR spectra (with quadrupole splittings of less than 10 kHz) were obtained on a Varian XL-100 spectrometer modified for wide sweeps, rf phase alternation and accurate short pulse intervals, operating in the FT mode at 15.36 MHz and at $29 \pm 1^\circ\text{C}$. Both normal (90°) and quadrupole echo ($90^\circ_x - \tau - 90^\circ_y - \tau - \text{echo}$) techniques were used. Wide band ^2H NMR spectra (with large quadrupole splittings) and ^{31}P NMR spectra were taken on a Bruker CXP-300 spectrometer operating in the FT mode at 46.06 MHz for ^2H and 121.39 MHz for ^{31}P , and at variable temperature. ^{13}C NMR spectra were recorded on a Varian CFT-20 spectrometer operating also in the FT mode at 20.10 MHz and $30 \pm 1^\circ\text{C}$. Centrifugation was performed on a Sorvall RC2-B centrifuge. Ultra-violet and visible (UV-VIS) absorption spectra were recorded on a Varian Cary 219 spectrophotometer. ESR spectra were obtained on a Varian E-9 spectrometer at room temperature ($22 \pm 2^\circ\text{C}$). Fatty acids were analyzed on a Hewlett-Packard 5710A gas chromatograph with a 15% DEGS (diethylglycolsuccinate) column at 250°C .

IV. RESULTS

A. Simulated ^2H NMR spectra in the presence of chemical exchange

Using the Fortran computer program (QUADENCH) given in Table 3, ^2H NMR spectra for a species in chemical exchange between a ^{partly} immobilized site giving rise to a 3 kHz quadrupole splitting and an isotropic site giving rise to a 20 Hz resonance have been calculated (Fig. 15). The spectra are shown for the cases of slow, intermediate and fast exchange. Both sites are equally populated ($p_A = p_B = 0.5$).

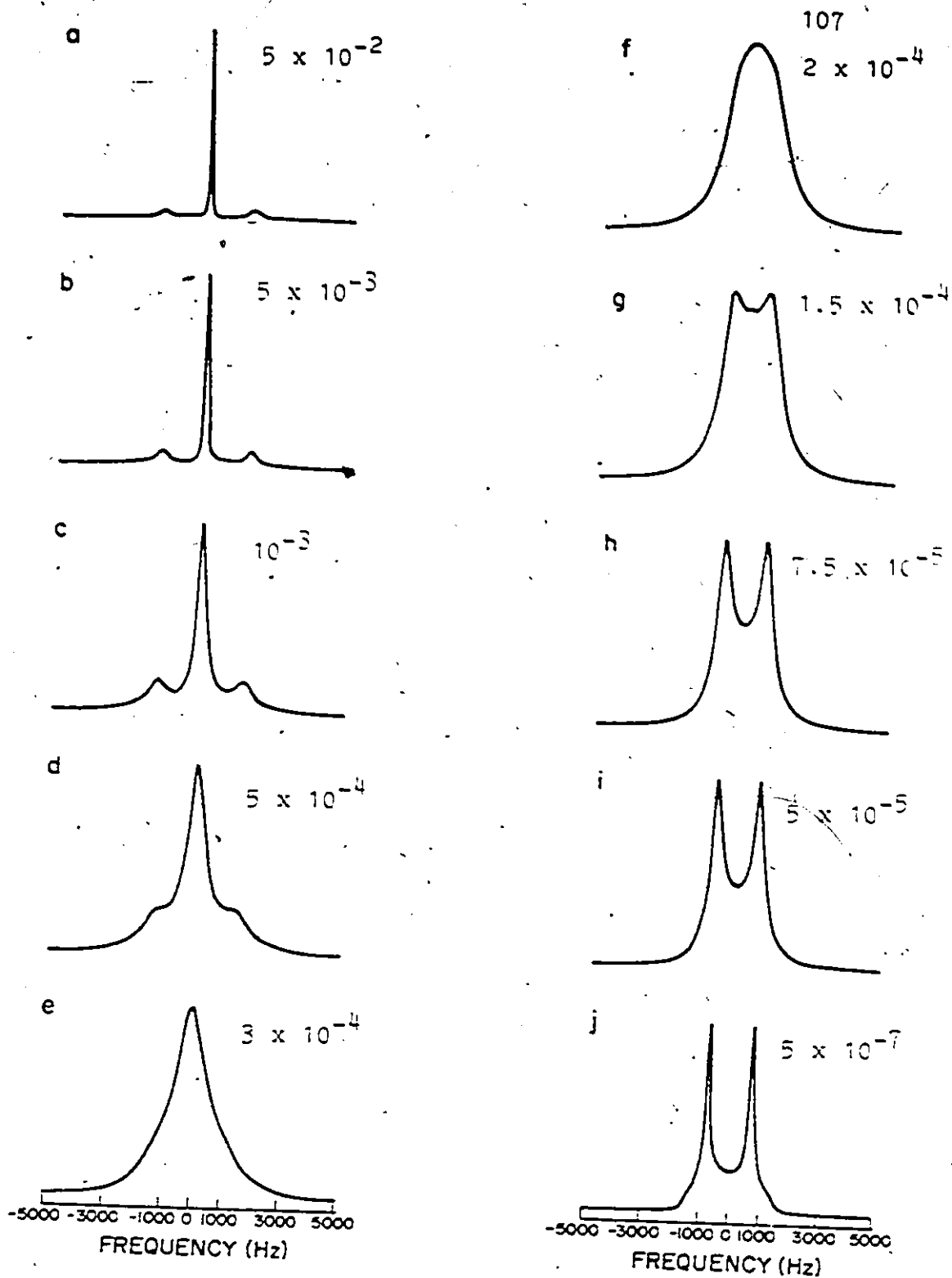


Fig. 15. ^2H NMR spectra resulting from the exchange between a powder pattern spectrum ($D' = 3$ kHz, $\Delta\nu_1' = 100$ Hz) and a single resonance ($\Delta\nu_2 = 20$ Hz) varying the residence times $\tau_1 = \tau_2$ indicated to the right of the spectra (in sec.). Spectra plotted at constant peak intensity.

B. Characterization of the system

(a) pK_a values

The pK_a values of the aromatic amino groups of local anesthetics PRC and TTC, as determined by ^{13}C NMR titration, and the pK_a values of the aromatic amino groups, as determined by acid-base titration, are given in Table 4. Fig. 16 shows the variation of the ^{13}C chemical shifts of the adjacent carbons with pH in the TTC molecule and Fig. 17 presents the titration curve of TTC. Note that the pK_a values of the tertiary amino groups were determined at 26 mM and that this value is concentration dependent (Table 4b). In the presence of PC multilamellar dispersions (26 mM), no change in the pK_a values of these local anesthetics was observed.

(b) Partition coefficients

The experimental partition coefficients of local anesthetics PRC and TTC between BPC buffer and phospholipid dispersions are listed in Table 5 for both low (5.5) and high (9.5) pH at room temperature. Three phospholipid dispersion systems were used, PC, PC-PS 1:1 and PC-PI 1:1. Except in the extreme cases (very

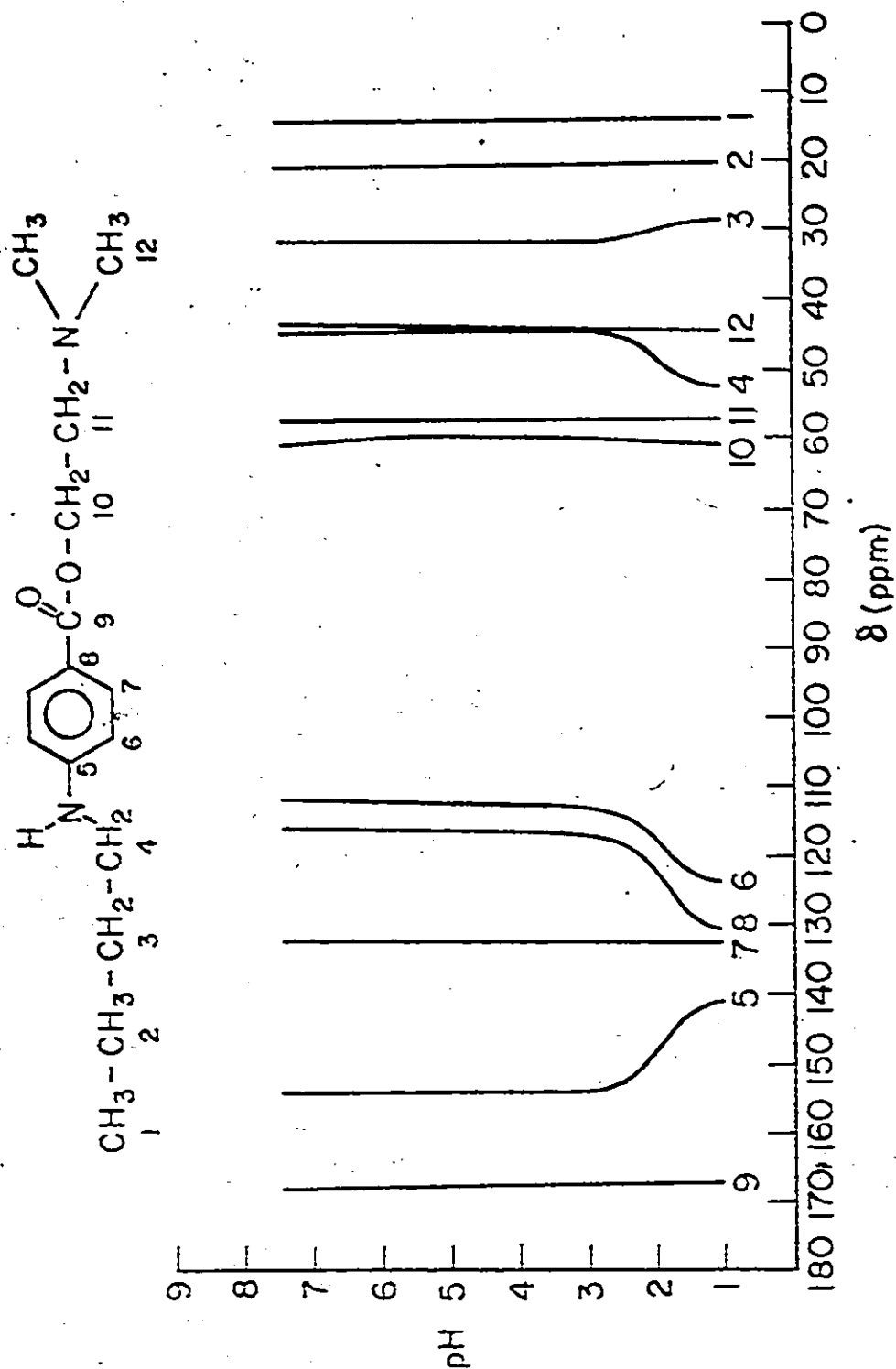


Fig. 16. Dependence of the ^{13}C NMR chemical shifts on pH for 0.5 M TTC. The pK_a is 1.95 for the aromatic amino group.

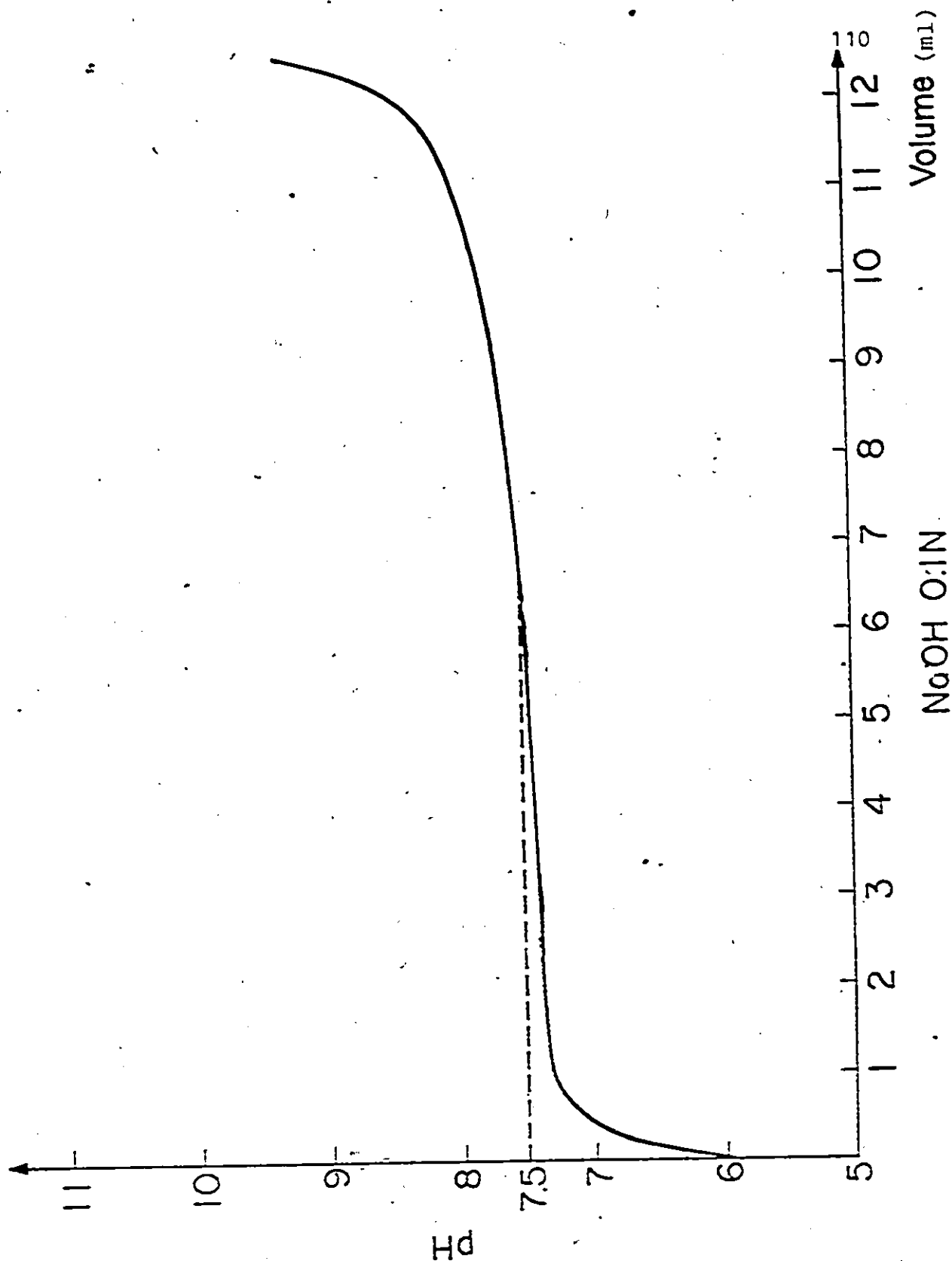


Fig. 17. Titration curve of 26 mM TTC (50 ml) with 0.1 N NaOH. The pK_a value is 7.5.

TABLE 4

pK_a values of local anesthetics PRC and TTC

(a)	Anesthetic	pK _a aromatic amino group ¹	pK _a tertiary amino group ²
	PRC	2.25	9.0
	TTC	1.95	7.5

(b)	Tertiary amino group	
Concentration (mM)	pK _a PRC	pK _a TTC
1	8.8	8.3 ³
10	8.9	7.8 ³
26	9.0	7.5
50	9.1	7.4

¹ 0.5 M in D₂O² 26 mM in BPC buffer³ Data from ref. 96

TABLE 5

Partition coefficients (K_p) for local
anesthetics PRC and TTC between SPC buffer
and phospholipids at different pH values

Lipid: Anesthetic	pH	K_p
PC-PRC	5.5	2.0
	9.5	45
PC-TTC	5.5	22
	9.5	660
PC-PS-PRC	5.5	8.0
	9.5	63
PC-PS-TTC	5.5	80
	9.5	550
PC-PI-TTC	5.5	60
	9.5	730

low or very high values), the errors on the K_p values were estimated at less than 10%. No variation in the measured K_p values was found upon decreasing the local anesthetic: lipid or water: lipid ratios by a factor of 10.

(c) Amount of local anesthetic bound and unbound

Knowing both the partition coefficient and the pK_a value of a local anesthetic in a given membrane: water system and assuming that those values do not change when the lipid concentration is increased, the percentage of charged and uncharged forms in water and in the membrane can be calculated using Eq. 37. Percentages obtained are given in Table 6 for PRC and TTC at pH 5.5 and 9.5, using NMR concentrations of lipid.

(d) Solubility of local anesthetics

The concentrations of saturated local anesthetic solutions in BPC buffer are given in Table 7 at different pH values and at room temperature. The solubility of local anesthetics follows their pK_a curve, the local anesthetic being much more soluble in its charged form.

TABLE 6

Percentages of local anesthetic in each form^a

System	[lipid](mM)	pH	Λ_w^+	AH_w^+	Λ_h^+	AH_h^+	A_{tL}
PC-PRC	52	5.5	0.03	93	0.07	7	7
		9.5	27	9	64	0.7	64
	260	5.5	0.02	71	0.3	28	29
		9.5	8	2	89	1	90
	650	5.5	0.02	50	0.5	50	50
		9.5	3	1	95	1	96
PC-TTC	52	5.5	0.5	53	14	33	47
		9.5	4	0.04	96	0.02	96
	260	5.5	0.2	18	24	57	81
		9.5	0.7	0.007	99	0.02	99
	650	5.5	0.08	8	27	64	92
		9.5	0.3	0.003	99	0.02	100

System	[lipid](mM)	pH	A_w^+	AH_w^+	A_i^+	AH_i^+	A_{ti}
PC-PS-PRC	52	5.5	0.02	76	0.08	24	24
		9.5	22	7	69	2	71
	260	5.5	0.01	38	0.2	61	61
		9.5	5	2	90	3	93
	650	5.5	0.006	20	0.3	80	80
		9.5	2	0.7	94	3	97
PC-PS-TTC	52	5.5	0.2	24	5	71	76
		9.5	4	0.04	96	0.1	96
	260	5.5	0.06	6	6	88	94
		9.5	0.9	0.009	99	0.1	99
	650	5.5	0.02	2	7	91	98
		9.5	0.4	0.004	99	0.1	99

^a AH_w^+ and AH_i^+ refer to the percentage of local anesthetic in the charged form in the water and lipid phase, respectively whereas A_w^+ and A_i^+ refer to the percentage of local anesthetic in the uncharged form, respectively. A_{ti} is the percentage of total anesthetic bound to the lipid phase.

TABLE 7

Solubility of local anesthetics in BPC buffer

pH	Anesthetic concentration (M)	
	FRC	TTC
5.5	2.6	1.4
6.0	2.4	1.4
6.5	2.5	1.4
7.0	2.5	1.0
7.5	2.4	0.014
8.0	0.45	0.0043
8.5	0.20	0.0015
9.0	0.029	0.0013
9.5	0.012	0.0012

(e) Fatty acid composition of phospholipids

Analysis of the fatty acid composition of the phospholipids used in the following experiments yielded the values shown in Table 8. These values are the average of three determinations.

(f) ESR results

The ESR spectra of PC multilamellar dispersions containing 16.7 mole% PC-5-SL display asymmetric resonances due to spin exchange interaction as shown in Fig. 18. The degree of asymmetry was evaluated by the ratio c/d shown in Fig. 18. The lower trace in Fig. 18 shows the effect of adding an equimolar amount of TTC to the lipid dispersions.

The c/d values are plotted as a function of TTC concentration in Fig. 19 at pH 5.5 and 7.8. Upon addition of TTC (equimolar to PC), the c/d ratio decreases by 44% at pH 7.8 and by 15% at pH 5.5.

Spectra recorded as a function of time at pH 5.5 showed that when TTC is added in the solid form after dispersion of the phospholipid, 70-80% of the effect has occurred within 20 min.; the complete equilibrium is attained after a period of 3-5 hours. When the anes-

TABLE 8

Fatty acid composition of phospholipids

Phospholipid	Percentage of fatty acids (% weight)							
	16:0	16:1	18:0	18:1	18:2	18:3	20:4	Others
Egg yolk PC	35.3	2.0	10.8	36.1	12.9			2.9
Egg yolk PE	20.2		27.5	17.2	7.5	6.2	16.9	4.5
Beef brain PS			39.4	41.2	1.2	11.7		7.2
Wheat germ PI	45.4			7.0	42.5	5.1		

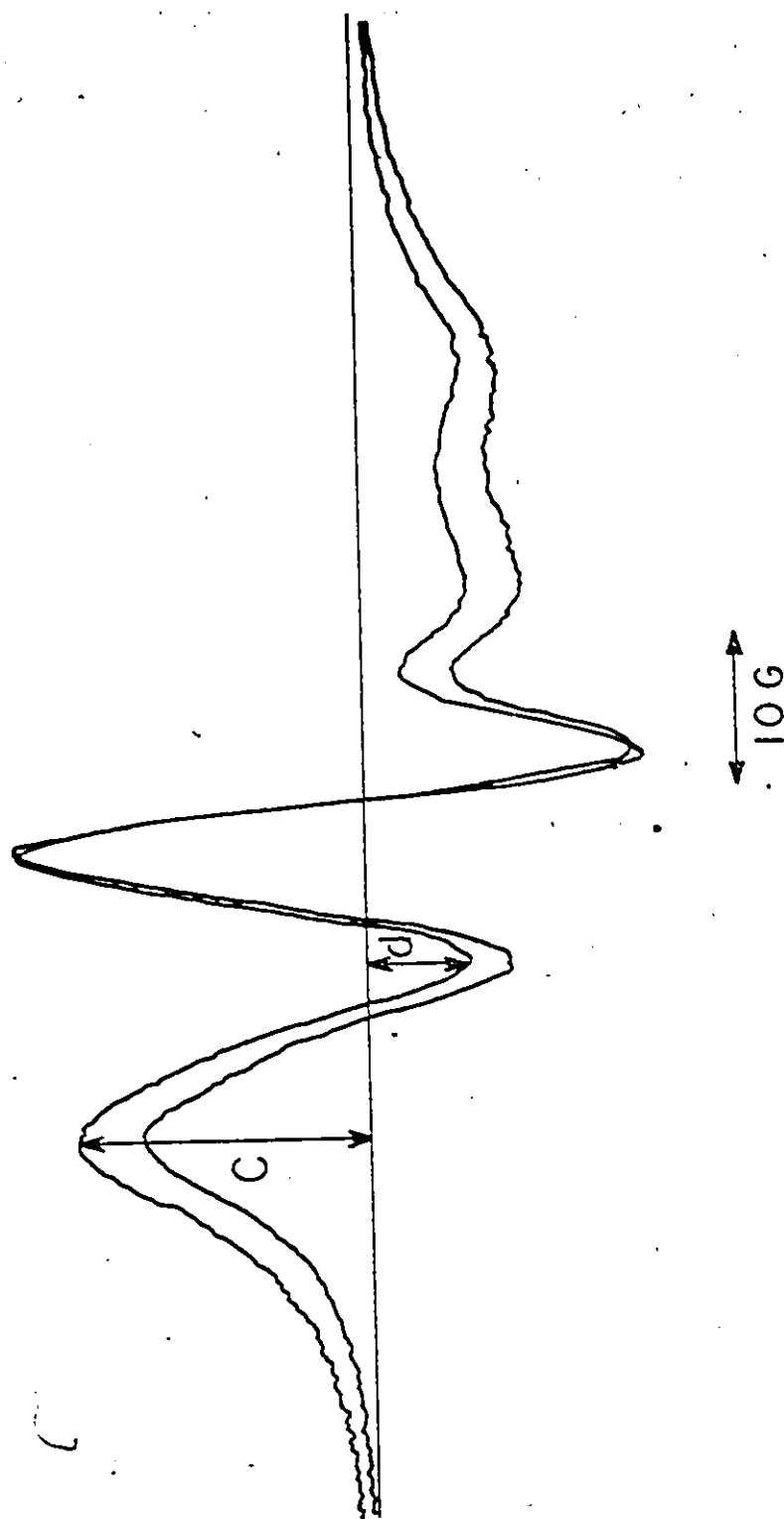


Fig. 18. ESR spectra (3.3 kG) of spin probe PC-5-SL (16.7 mole%) in PC dispersions at pH 7.8. Upper trace, no TTC; lower trace, equimolar concentrations of PC and TTC. The ratio of the heights c/d is a measure of the spin-exchange interaction.

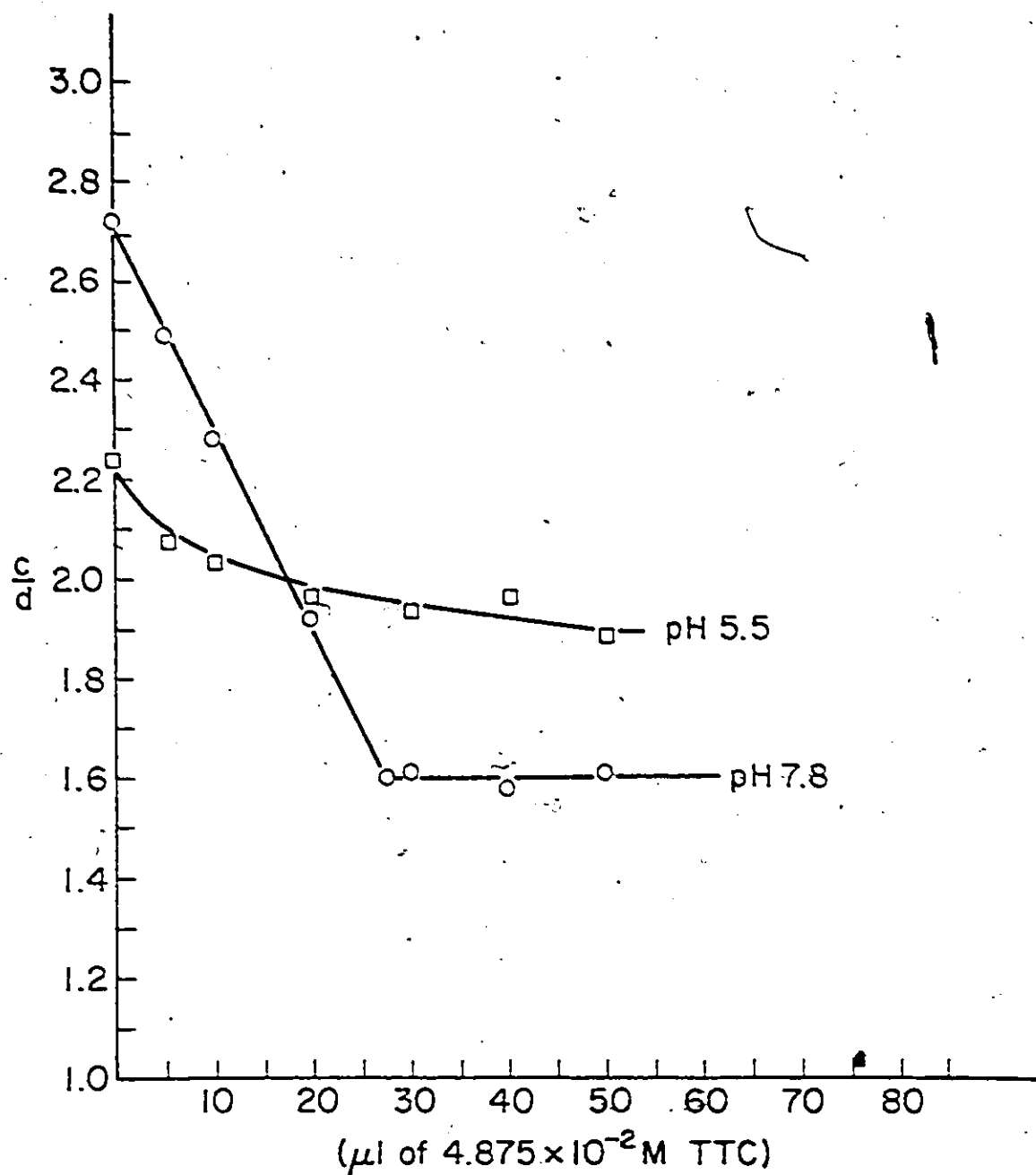


Fig. 19. c/d ratio in the ESR spectra of PC-5-SL in PC dispersions as a function of TTC concentration at pH 5.5 (12 mole% PC-5-SL) and at pH 7.8 (16.7 mole% PC-5-SL). At 40 μl TTC, the concentrations of PC and TTC are equal.

thetic is added before lipid dispersion, complete equilibrium was reached before 15 minutes. At pH 9.5, no delay time to reach equilibrium has been observed in either method of sample preparation. In the presence of PS, under any conditions, the equilibrium was reached within a period of 15 minutes.

(g) Hydrolysis of local anesthetic and DPPC

^2H NMR spectra of TTC- d_6 in PC dispersions at pH 9.5 showed no change during a period of 36 hours at 30°C . The pH value remained unchanged after the experiment. The extent of hydrolysis was thus too low to affect the spectra significantly.

When the hydrolysis of DPPC was monitored by TLC at 47°C , a trace of lyso PC was detected at pH 9.5. For all lower pH values, no sign of hydrolysis was evident for a period of 10 hours.

(h) Light scattering

Upon addition of TTC, the light scattering measured for PC-PS multilamellar dispersions was unchanged (within experimental error) under any condition of pH

or anesthetic concentration. The local anesthetic
does not alter the size of the phospholipid dispersions.

C. ^2H NMR results

(a) Deuterated local anesthetics in PC dispersions

i. Linewidth and quadrupole splitting values

Table 9 presents the quadrupole splitting or linewidth values for the five specifically-deuterated local anesthetic molecules in PC multilamellar dispersions under several lipid concentrations and at pH 5.5 and 9.5. When both a quadrupole splitting and a narrow resonance are present, only the quadrupole splitting value is given. Representative spectra of the three different deuterated TTC at low and high pH are shown in Fig. 20.

ii. Variation of the lipid: water ratio

The spectrum of TTC- d_6 in PC multilamellar dispersions displays a broad central resonance and a quadrupole splitting. Addition of water did not cause any variation in the quadrupole splitting but caused a decrease of the linewidth of the central resonance. This is illustrated in Fig. 21a for TTC- d_6 at pH 5.5 and after freeze-thawing of the sample in Fig. 21b. The variation of the linewidth of the central resonance

TABLE 9

Linewidths (Hz) and quadrupole splittings¹ (Hz, numbers underlined)
for deuterated local anesthetics in aqueous
dispersions of egg PC (1.0 ml BPC buffer)

		pH = 5.5		pH = 9.5	
		[Lipid](mM)		[Anesthetic](mM)	
PRC-d ₄		4.6	5.5	120	6.0
				29	<u>1520</u>
PRC-d ₂		5.5	12	10	31
					<u>1400</u>
TTC-d ₆		2.0	136	<u>1840</u>	6.0
					<u>980</u>
TTC-d ₂		9.0	400	<u>13400</u>	10
					<u>12100</u>
TTC-d ₃		3.0	130	200	9.5
					<u>880</u>

¹ All spectra containing a quadrupole splitting also manifest a narrower resonance for the anesthetic in water, in fast exchange with the anesthetic weakly-bound to the lipid phase.

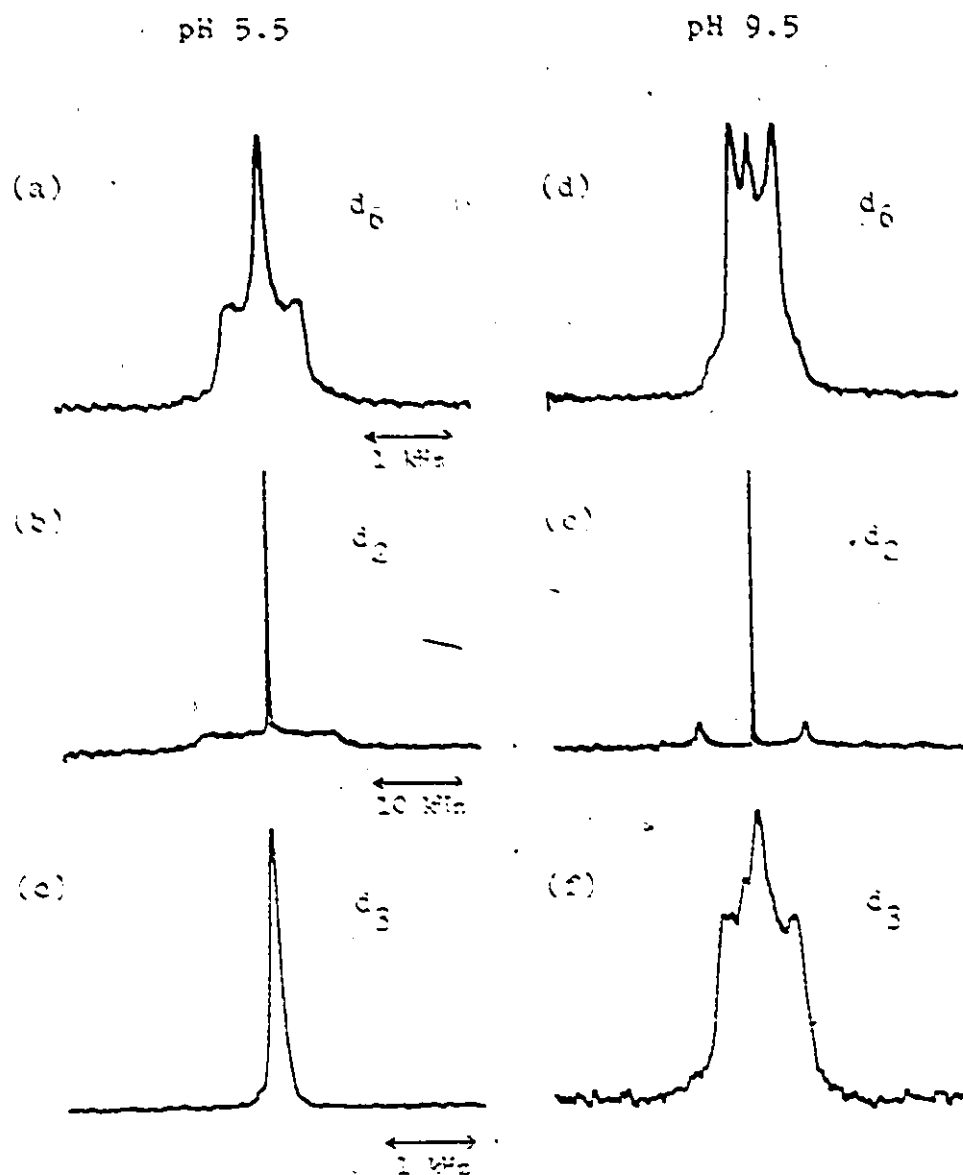


Fig. 20. ^2H NMR spectra of (a) 65 mM TTC- d_6 in 275 mM PC, pH 5.5, (b) 65 mM TTC- d_2 in 650 mM PC, pH 5.5, (c) 26 mM TTC- d_3 in 52 mM PC, pH 5.5, (d) 65 mM TTC- d_6 in 275 mM PC, pH 9.5, (e) 65 mM TTC- d_2 in 650 mM PC, pH 9.5 and (f) 26 mM TTC- d_3 in 52 mM PC, pH 9.5. The samples were run in BPC-buffer at 30°C . The spectra were acquired with the quadrupole echo technique ($\tau_1 = 60 \mu\text{s}$, $\tau_2 = 50 \mu\text{s}$), a 0.2 s recycle time, 3000-10,000 scans and a 90° pulse of 4-5 μs .

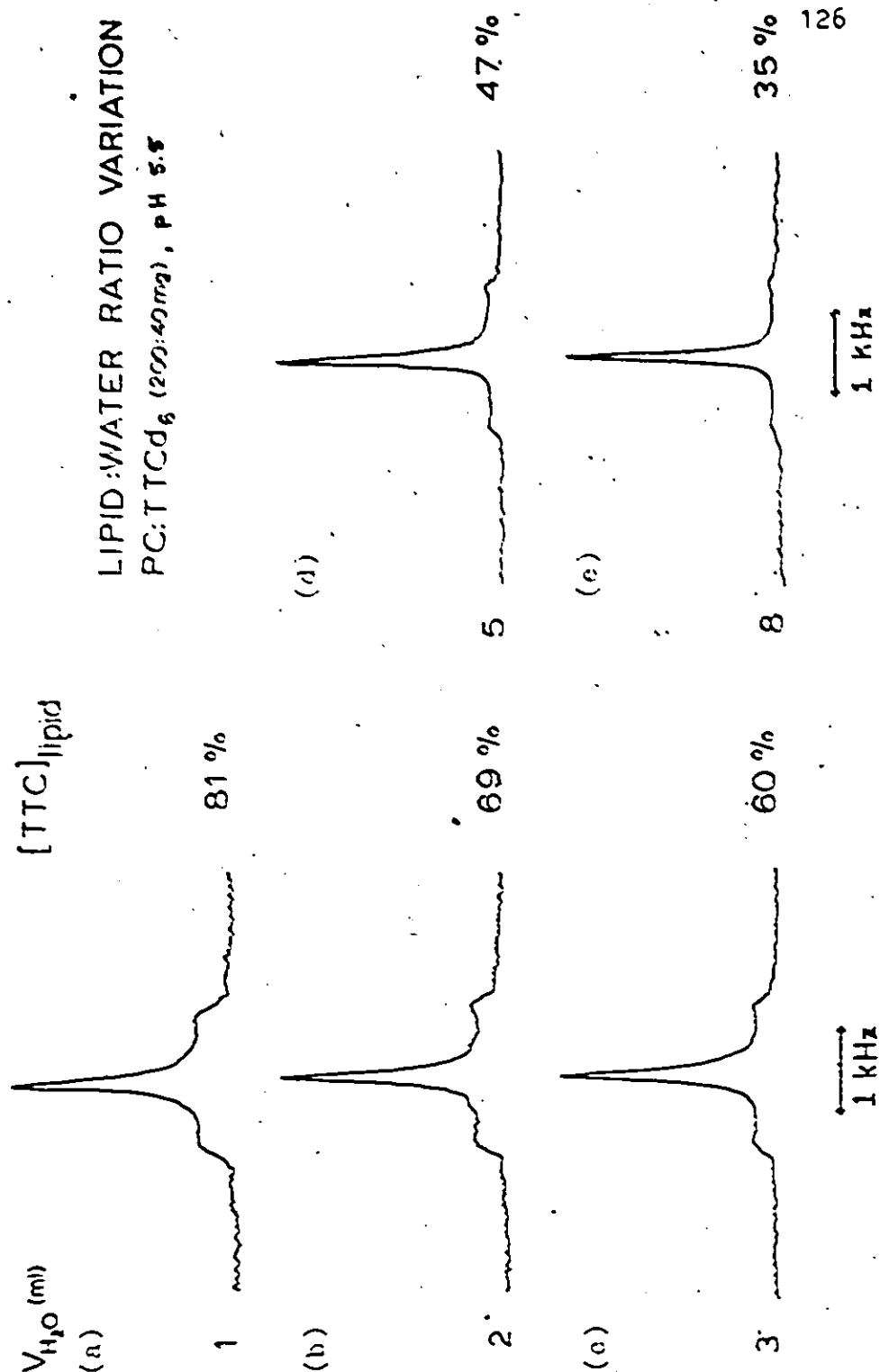


Fig. 21a. ²H NMR spectra (15.4 MHz) of TTC-d₆ (52 mmol) at different lipid water ratios, pH 5.5. The samples consisted of 260 mmol PC and (a) 1 ml, (b) 2 ml, (c) 3 ml, (d) 5 ml and (e) 8 ml BPC buffer. The number to the right of the spectra represent the percentage of total anesthetic bound. Spectra were acquired with the quadrupole echo technique ($\tau_1 = \tau_2 = 100 \mu s$) and folding about the central frequency of the spectrum, the recycle time was 0.2 s, the temperature 29°C and the 90° pulse 10 μs .

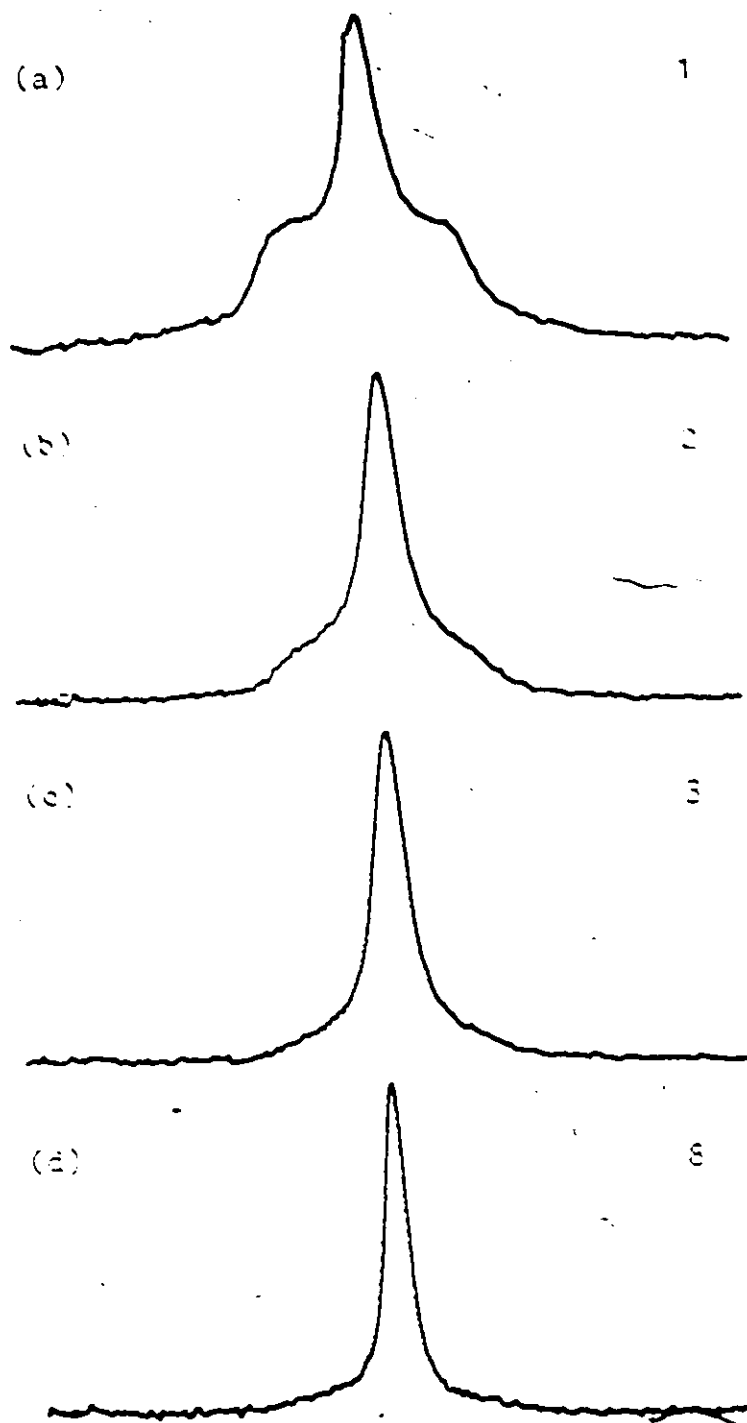


Fig. 21b. ^2H NMR spectra (46.06 MHz) of TTC- d_2 (52 mmol) at different lipid: water ratios, pH 5.5. The samples consisted of 260 mmol PC and (a) 1 ml, (b) 2 ml, (c) 3 ml and (d) 8 ml BPC buffer. The conditions are identical to those in Fig. 21a except that the samples were freeze-thawed three times before running. The spectra were acquired with the quadrupole echo technique, a 0.2 s recycle time, 3000 scans, a 100 kHz sweep width and a 90° pulse of 6 μs .

is plotted as a function of PC concentration in Fig. 22.

iii. Simulated spectra

Using the Fortran program in Table 3, simulations of the spectra in Fig. 21a were attempted assuming an intermediate exchange between two sites (Fig. 23) and a three site exchange (Fig. 24) where two sites are in fast exchange and one is in slow exchange with the two others.

In Fig. 23, the spectrum (e) was first simulated to fit the spectrum (e) of Fig. 21a. An exchange between a sharp line ($\Delta\nu_1 = 8$ Hz) and a 1640 Hz quadrupole splitting ($\Delta\nu_2 = 50$ Hz) was assumed with residence times proportional to the percentage of anesthetic bound and in the water phase ($\tau = 4 \times 3 \times 10^{-5}$ sec.). The spectral width was 5000 Hz, the number of points 500 and the quadrupole coupling constant 170 kHz. The four other spectra (Fig. 23 a-d) were calculated were calculated in a similar way, varying the residence times to account for the variations in the percentage of anesthetic bound as given in Fig. 21a.

The spectra of Fig. 24 were calculated assuming

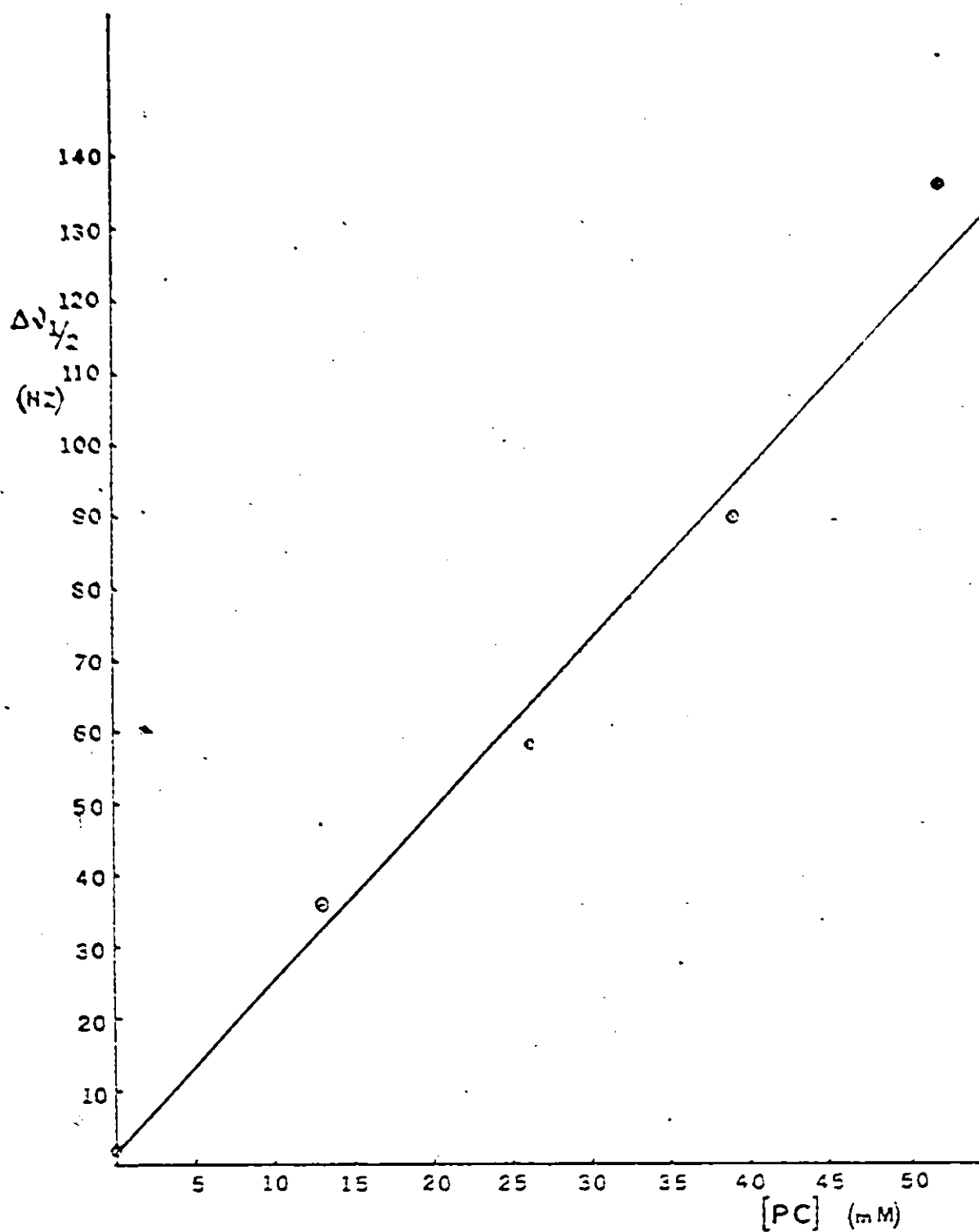


Fig. 11. Variation of ^2H NMR linewidth vs PC concentration for 26 mM TTC- d_6 at pH 5.5.

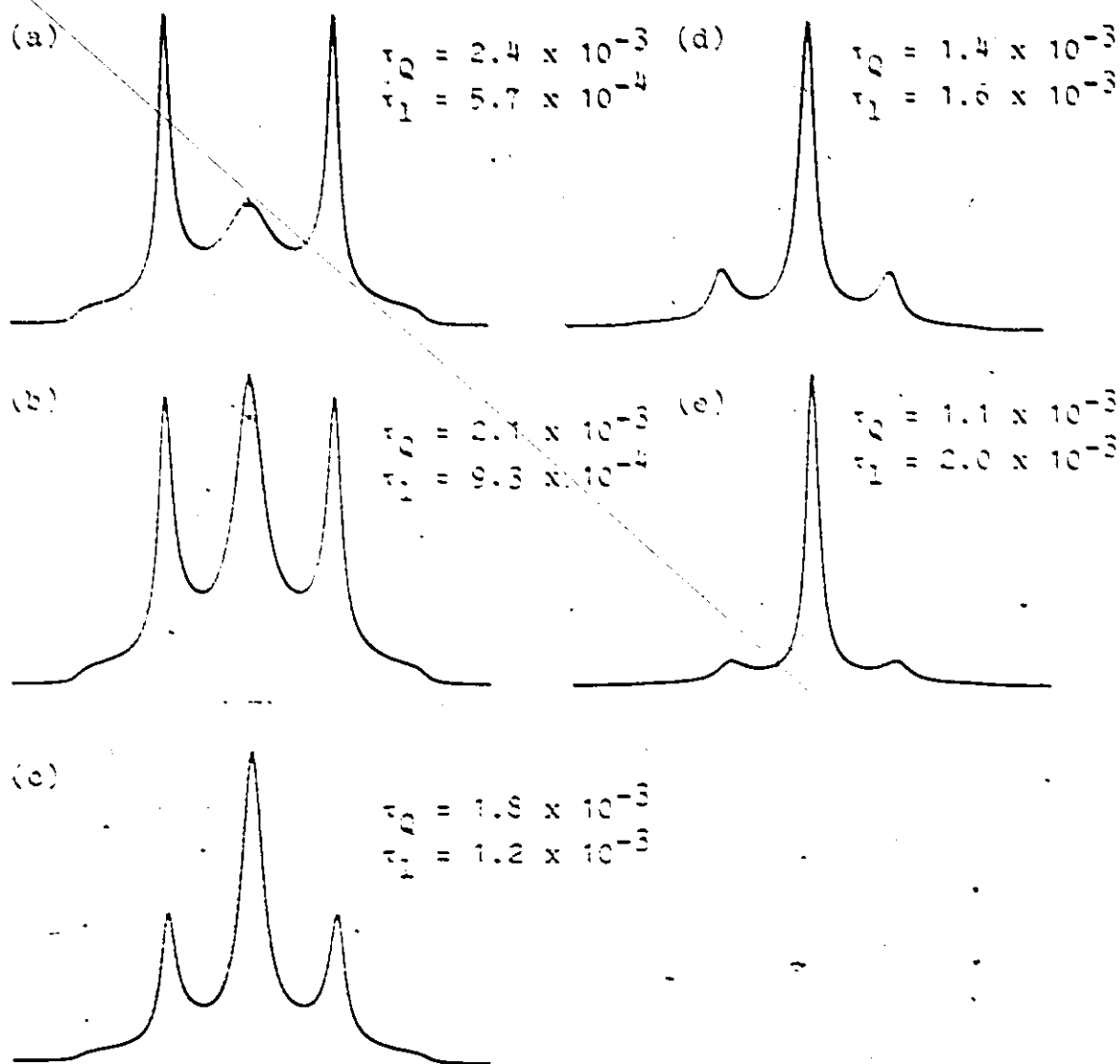


Fig. 23. Calculated ^2H NMR spectra resulting from the exchange between a single line ($\Delta\nu_1 = 8$ Hz) and a quadrupole splitting ($D_Q = 1840$ Hz, $\Delta\nu_1 = 50$ Hz) in an intermediate rate of exchange. The values to the right of the spectra indicate the residence times of the quadrupole splitting (τ_Q) and of the single line (τ_1).

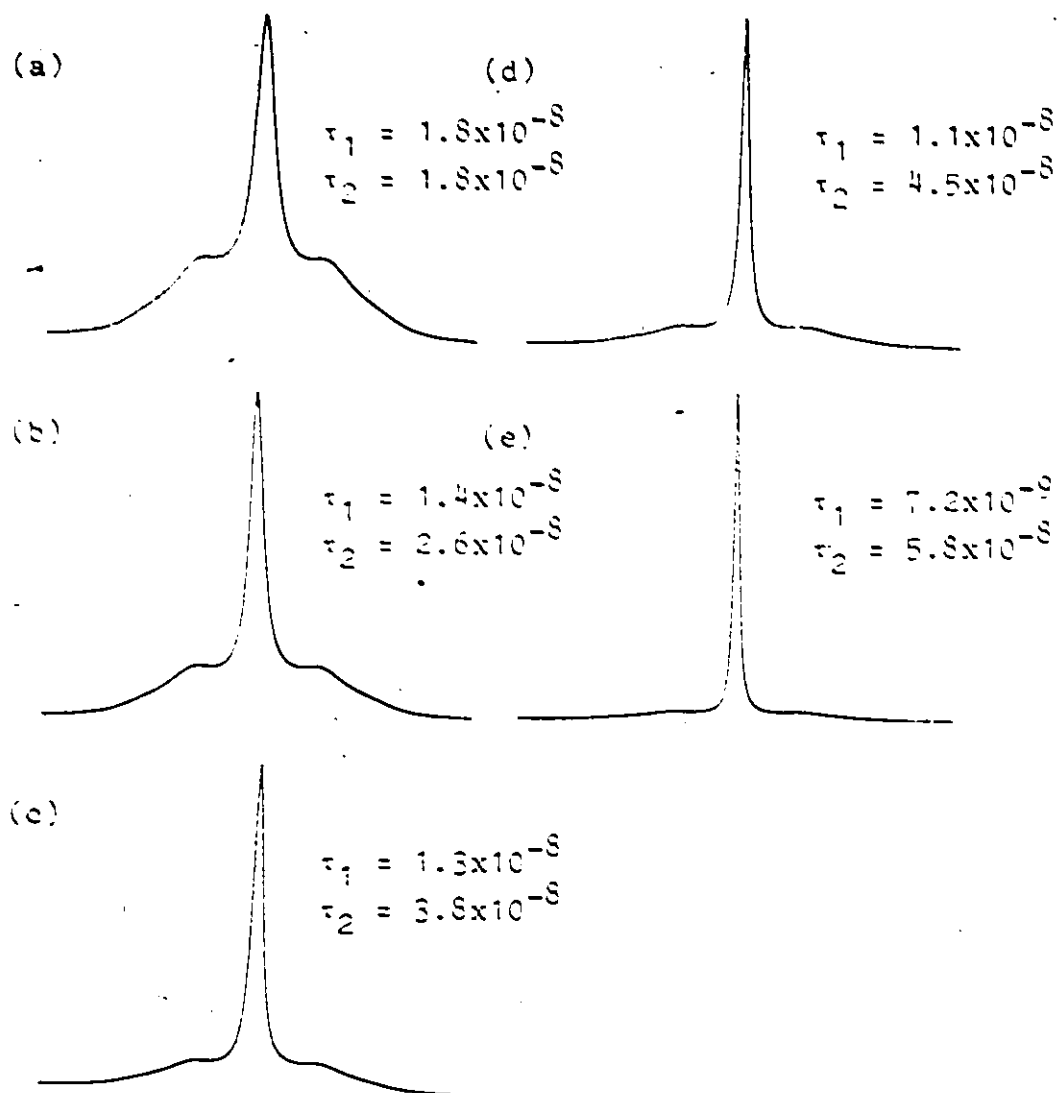


Fig. 24. Calculated ^2H NMR spectra resulting from the addition (slow exchange) of a quadrupole splitting ($D_Q = 1840$ Hz, $\Delta\nu_Q^c = 500$ Hz, $\Delta\nu_Q^i = 0$) to the spectrum resulting from the fast exchange² between a broad line ($\Delta\nu_i = 660$ Hz) and a narrow line ($\Delta\nu_i = 10$ Hz) in different proportions corresponding to the conditions of Fig. 21a. The values of τ_1 (broad line) and τ_2 (narrow line) are indicated to the right of the spectra.

that the 1840 Hz quadrupole splitting ($\Delta\nu_{\frac{1}{2}} = 600$ Hz) is in slow exchange with the broad central line. Its intensity was determined from the integration of the spectra in Fig. 21a. The central resonance was assumed to result from the fast exchange ($\tau = 10^{-8}$ sec.) between a 660 Hz line ($D_q = 60$ Hz, $\Delta\nu_{\frac{1}{2}}^o = 300$ Hz) and a 10 Hz line whose proportions ($\tau = 4 \times 10^{-9}$ sec.) were calculated assuming a partition coefficient of 5 between the lipid and water phases. The spectral width was 6000 Hz, the number of points 500 and the quadrupole coupling constant 170 kHz.

(b) Deuterated local anesthetics in PC-PS dispersions

i. Linewidth and quadrupole splitting values

Table 10 gives the ^2H NMR linewidths or quadrupole splittings observed for deuterated local anesthetics PRC and TTC in PC-PS multilamellar dispersions at pH 5.5 and 9.5. All spectra showing a quadrupole splitting also display a central broad resonance whose value is not indicated in Table 10. The spectra of specifically-deuterated TTC at low (5.5) and high (9.5) pH are shown in Fig. 25. When pH was increased, the signal of TTC- d_3 changed from a single resonance to a quadrupole splitting whereas the converse was observed for TTC- d_6 .

ii. Variation of the lipid: water ratio

When the ratio of the two phases present, lipid and water was modified, the quadrupole splittings of all deuterated local anesthetics remain constant. Under the same conditions, the linewidths of the central resonances change. Linewidths are plotted in Fig. 26 as a function of lipid concentration.

TABLE 10

Values of ^2H NMR quadrupole splittings D_Q (Hz) and linewidths $\Delta\nu_{1/2}$ (Hz) for deuterated local anesthetics in PC-PS multilamellar dispersion

Anesthetic ¹	pH 5.5	pH 9.5
PRC-d ₂	sl ²	sl ²
PRC-d ₄	sl ²	910
TTC-d ₂	14600	12600
TTC-d ₃	sl ²	550
TTC-d ₆	1800	sl ²
TTC-d ₆ ³	1560	sl ²

¹ The molar ratio PC:PS:anesthetic was 1:1:1. The quantity of total lipid ranged from 52 mM to 260 mM depending on the partition coefficient.

² sl means that a single resonance was observed but the width was not measured because of the inaccuracy resulting from the folding of the spectrum about the central carrier frequency.

³ Data obtained with PC-PI multilamellar dispersions.

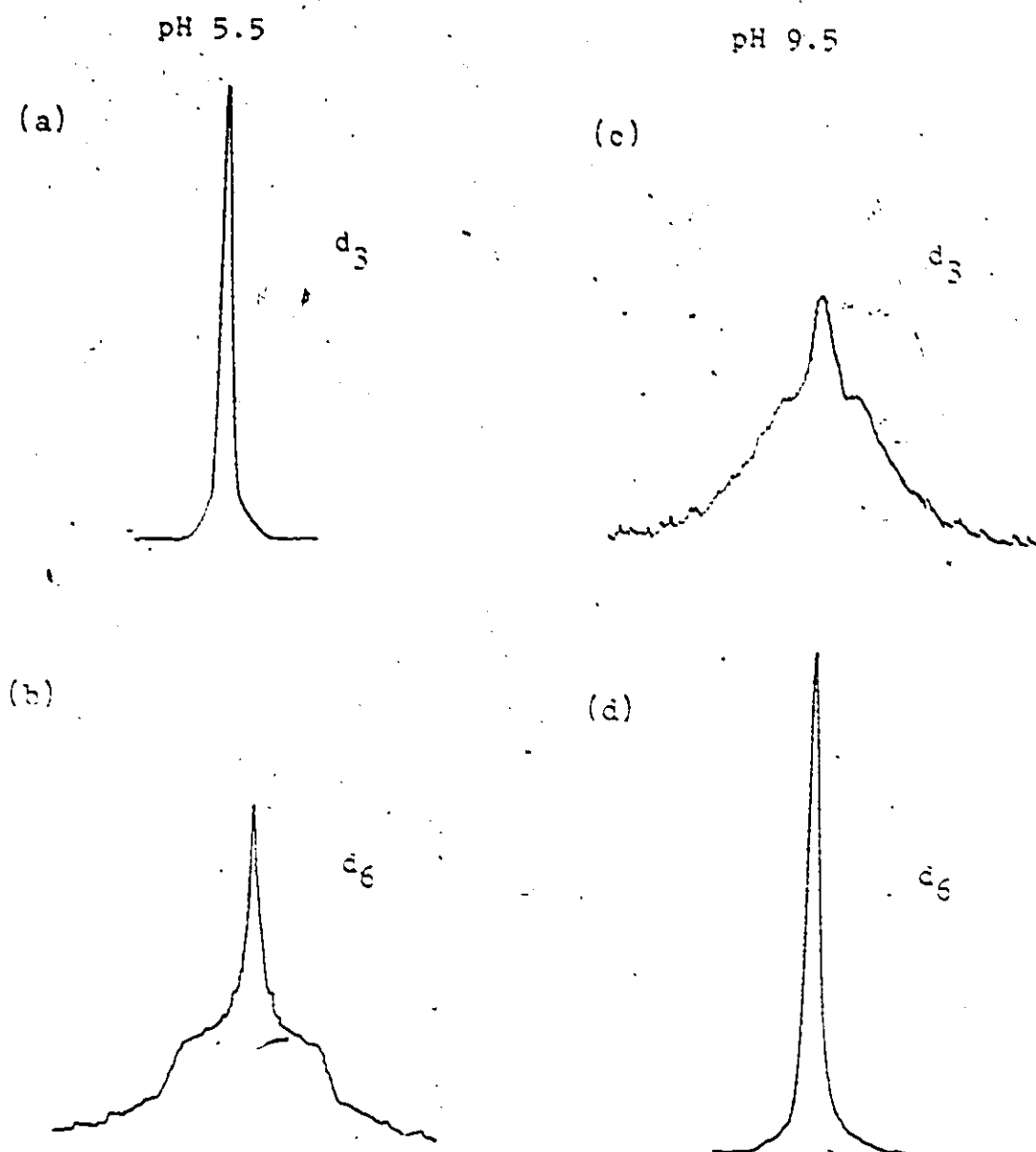


Fig. 25. ^2H NMR spectra (15.4 MHz) of 26 mM deuterated local anesthetic in 52 mM PC-PS 1:1 multilamellar dispersions in BPC buffer at 30°C (a) TTC- d_3 pH 5.5, (b) TTC- d_6 pH 5.5, (c) TTC- d_3 pH 9.5 and (d) TTC- d_6 pH 9.5. The spectra were obtained with the quadrupole echo technique ($\tau_1 = \tau_2 = 100$ μs) with folding about the central carrier frequency. The acquisition temperature was 29°C, the number of scans 1000-30,000 and the 90° pulse 19 μs .

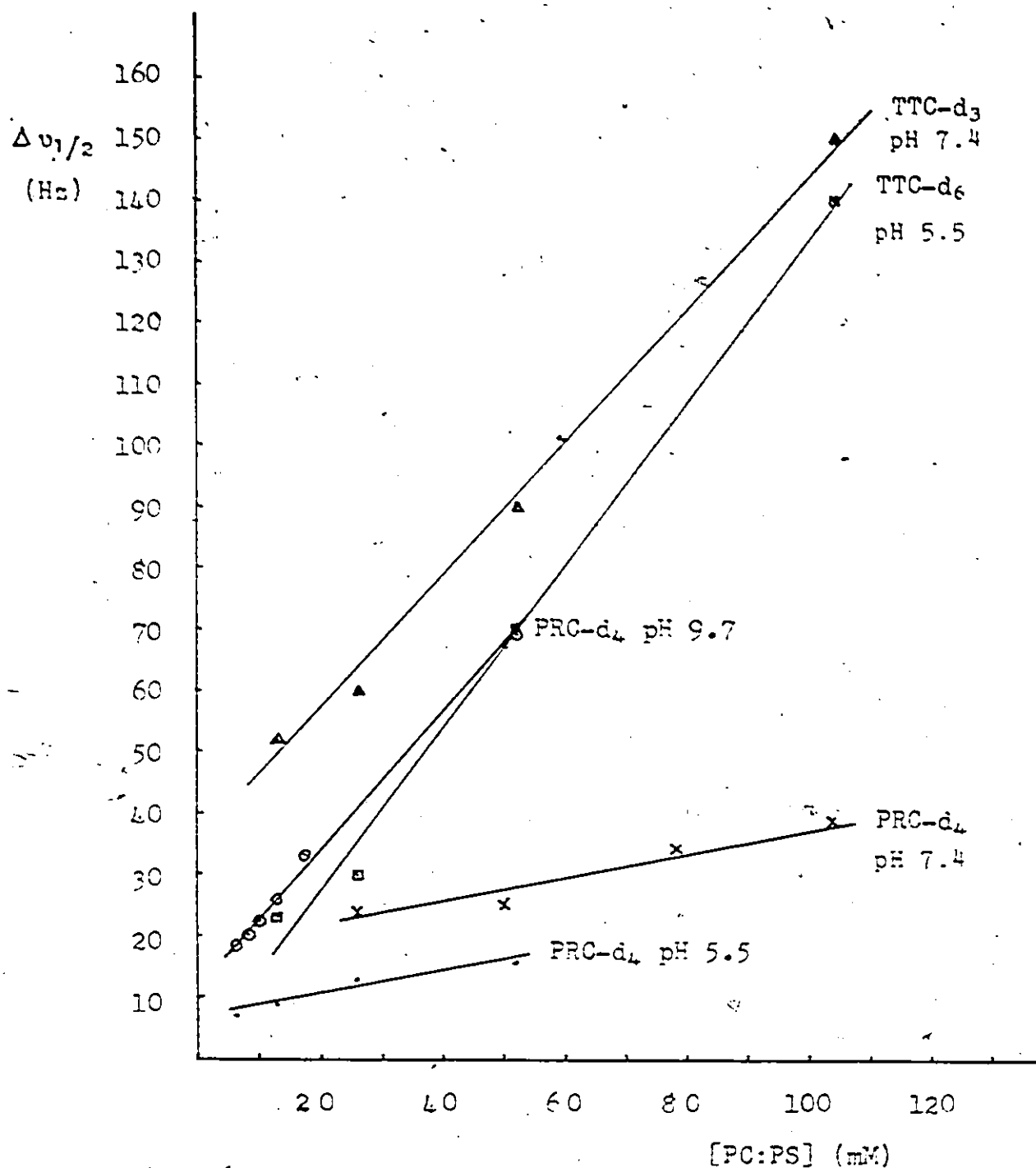


Fig. 26. Variation of ^2H NMR linewidths ($\Delta \nu_{1/2}$) as a function of phospholipid PC-PS concentration. Samples were run at 30°C in phosphate buffer. (•) PRC-d₄ pH 5.5, (×) PRC-d₄ pH 7.4, (◻) TTC-d₆ pH 5.5, (○) PRC-d₄ pH 9.7 and (Δ) TTC-d₃ pH 7.4.

(c) Local anesthetics in deuterated PC dispersions

i. Effect on perdeuterated DPPC (DPPC- d_{62})

The spectrum of DPPC- d_{62} dispersions is the summation of the spectra of all the resonances of the deuterons of the fatty acyl chains. Most of the peaks of the resulting envelope can be only tentatively assigned (94). Addition of local anesthetic produces a drastic change in the shape of this envelope (Fig. 27). Although the width of the pattern does not decrease much, the intensity increases towards the center at the expense of the edges. This seems to indicate a decrease of most quadrupole splittings, but to different extents for each. No assignment of peaks is possible in the anesthetic-perturbed spectrum. The effect of both anesthetics at both pH 5.5 and 9.0 is qualitatively similar. The amount of local anesthetic required to produce the same effect is of course anesthetic and pH dependent. Similar results were obtained for the DPPC- d_{62} -PS system.

ii. Effect on specifically-deuterated DPPC

In order to analyze the results obtained with

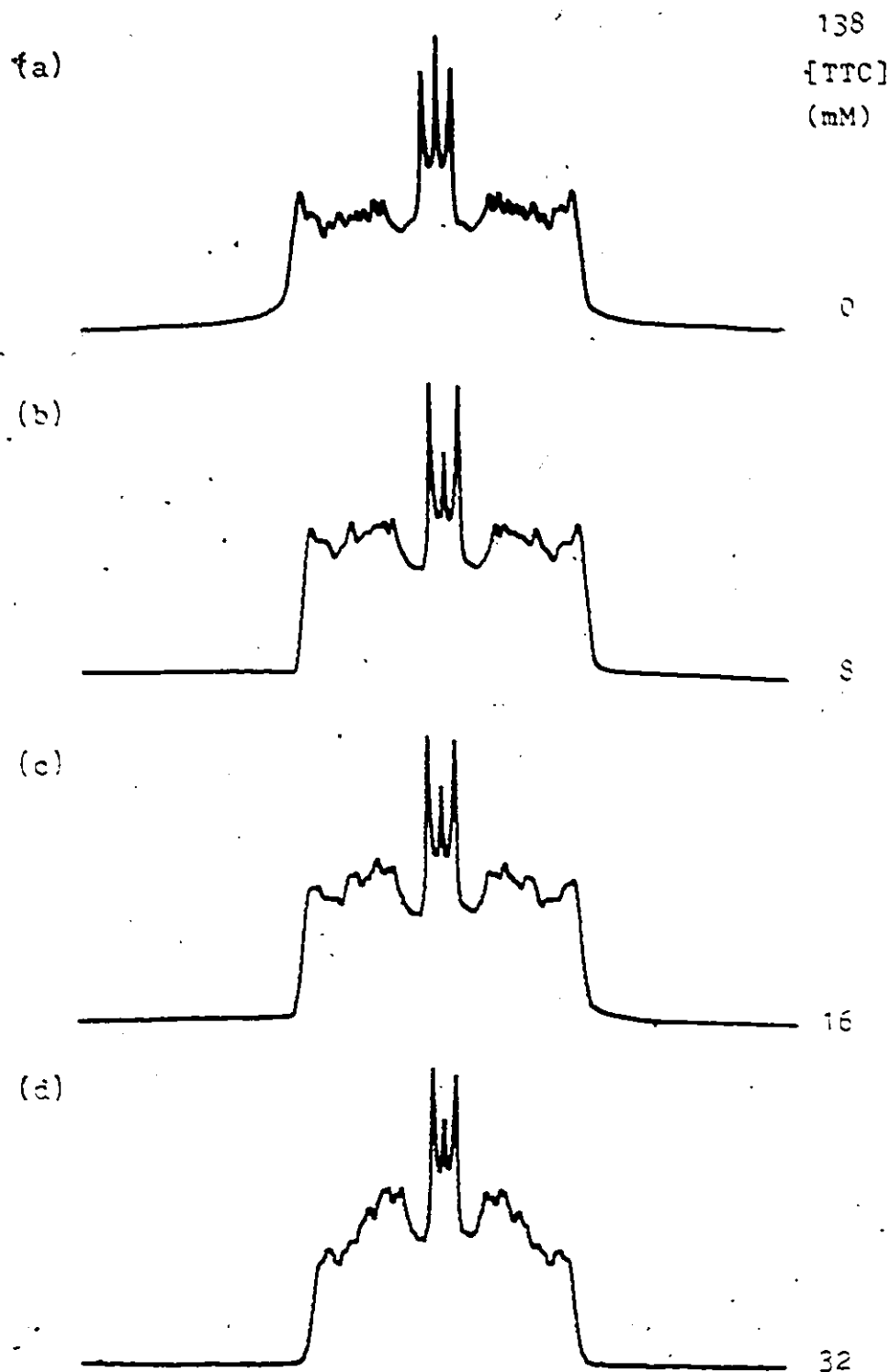


Fig. 27. ^2H NMR spectra (46.06 MHz) of 65 mM DPPC- d_{62} multilamellar dispersions in EPC buffer at pH 5.5 with (a) no TTC, (b) 8 mM TTC, (c) 16 mM TTC and (d) 32 mM TTC. The spectra were recorded at 47°C using the quadrupole echo technique ($\tau_1 = 60 \mu\text{s}$, $\tau_2 = 50 \mu\text{s}$). The recycle time was 0.2 s, the spectral width 100 kHz and the number of data points 4096, the 90° pulse 5 μs and the number of scans 4000.

DPPC- d_{62} , specifically chain-deuterated DPPC dispersions were used. Fig. 28 a-c shows the effect of TTC on the spectrum of DPPC- $12'-d_2$ at pH 5.5 and 47°C. In Fig. 28 d-f is also shown the effect of TTC on the spectrum of PC- d_{44} at pH 5.5 and 30°C. The variations of the quadrupole splittings (D_Q) of specifically-deuterated PC are plotted in Figs. 29 and 30 as a function of concentration of TTC bound (calculated from partition coefficients) at pH 5.5 and 9.0, respectively. These variations can be normalized to a common starting point by plotting D_Q/D_{Q0} as a function of concentration of TTC bound (Figs. 31 and 32), where D_{Q0} is the quadrupole splitting of the phospholipid before addition of anesthetic.

The spectra of PC- d_{44} -PS 1:1 dispersions are shown in Fig. 33 at low and high pH in the presence of variable amounts of TTC at 30°C. The dependence of the D_Q/D_{Q0} values of PC- d_{44} -PS and PC- d_9 -PS are plotted in Fig. 34 as a function of the concentration of TTC bound.

LABELLED CHAIN (2)

LABELLED HEAD GROUP

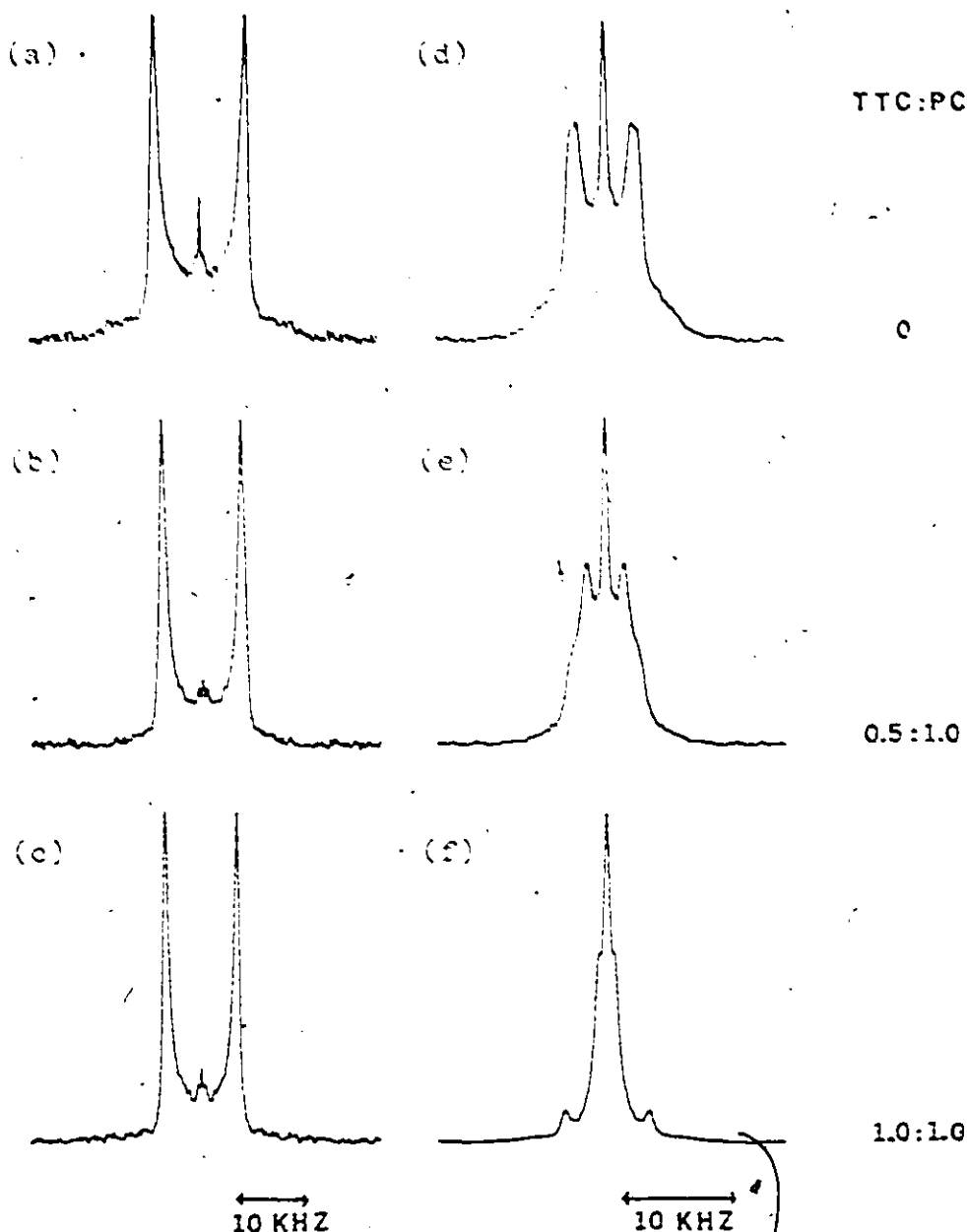
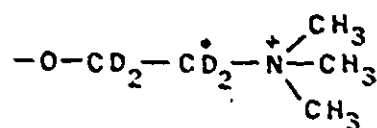
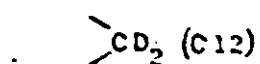


Fig. 28. ^2H NMR spectra (46.06 MHz) of (a-c) PC-12'-d₂, 47°C and (d-f) PC-d₄, 30°C. The mole ratio is indicated to the right of the spectra. All spectra were obtained at pH 5.5 in BPC buffer (32 mM lipid in 1.0 ml buffer). The spectra were acquired with the quadrupole echo technique ($\tau_1 = 60 \mu\text{s}$, $\tau_2 = 50 \mu\text{s}$), 2048 data points, a 500 kHz spectral width, $\tau_2 = 4.8 \mu\text{s}$ (PC-12'-d₂) or $8 \mu\text{s}$ (PC-d₄) 90° pulse, 50,000 (PC-12'-d₂) or 15,000 (PC-d₄) scans and a 0.08 s (PC-12'-d₂) or 0.2 s (PC-d₄) recycle time.

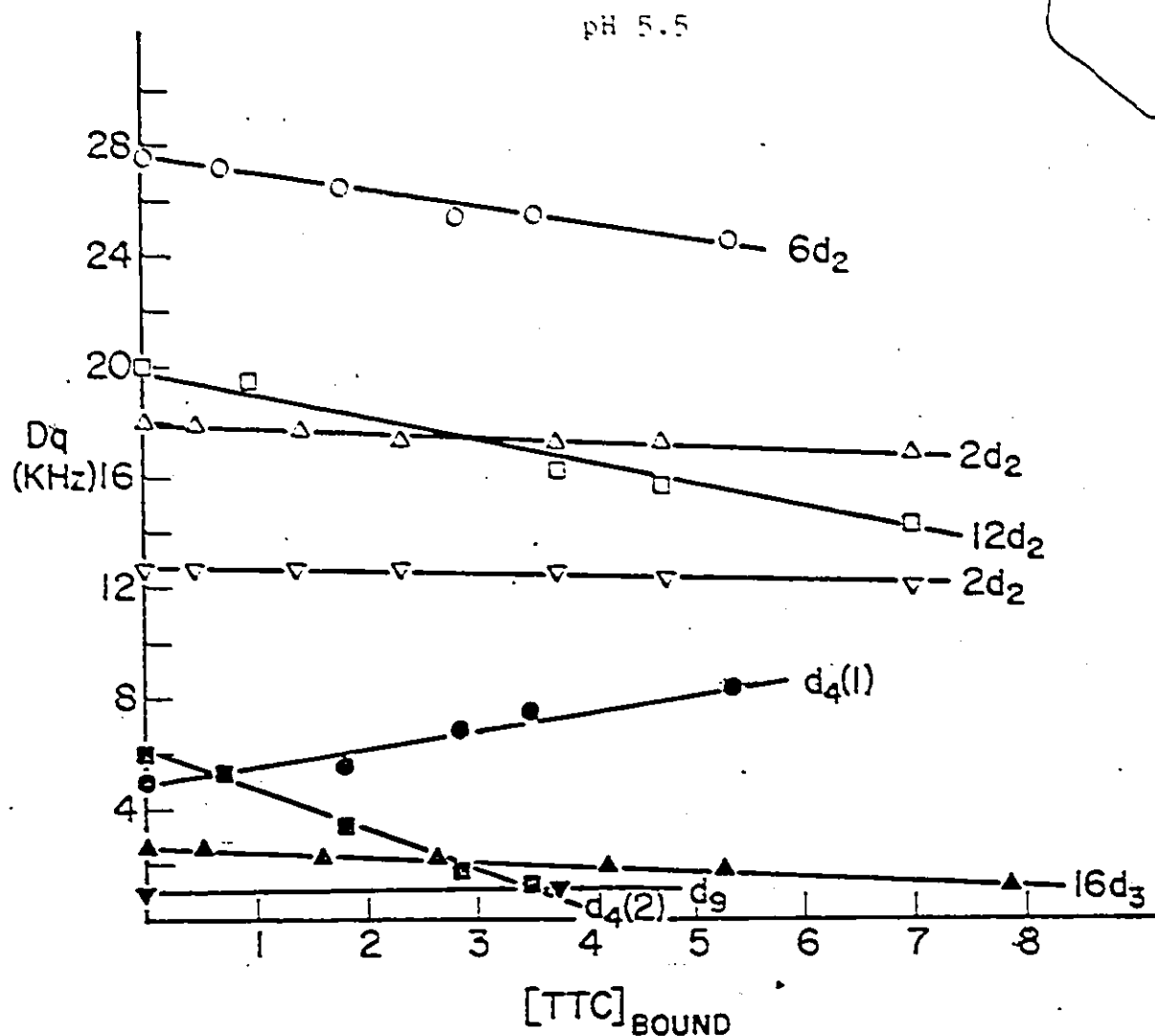


Fig. 29. Graph of quadrupole splittings (D_q) of deuterated PC dispersions as a function of the amount of TTC bound at pH 5.5 (each unit corresponds to 0.05 mole TTC/mole PC). Chain deuterated PC dispersions consisted of saturated molecules (DPPC-2',6',12'-d₂ and DPPC-16'-d₃) whereas head group deuterated PC molecules are from natural source (PC-d₄ : egg PC and PC-d₉ : egg PE). The spectra were acquired at 47°C for DPPC samples and at 30°C for unsaturated PC samples.

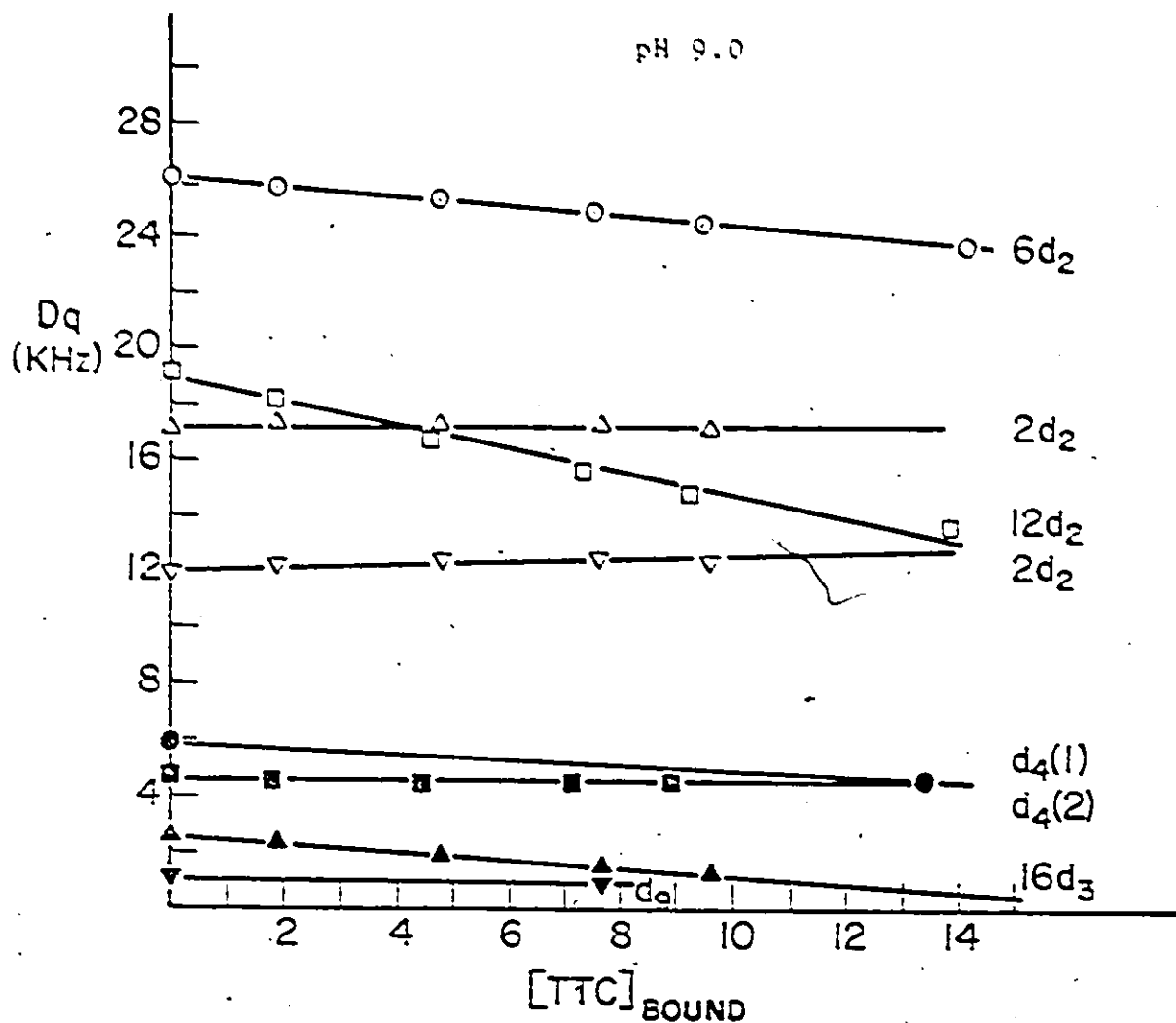


Fig. 30. Graph of quadrupole splittings (D_q) of deuterated PC dispersions as a function of the amount of TTC bound at pH 9.0 (each unit corresponds to 0.05 mole TTC/mole PC). Chain deuterated PC dispersions consist of saturated molecules (DPPC-2', 6', 12'- d_2 and DPPC-16'- d_2) whereas head group deuterated PC dispersions are from natural source (PC- d_4 : egg PC, PC- d_0 : egg PE). The spectra were acquired at 47°C for DPPC samples and at 30°C for saturated PC samples.

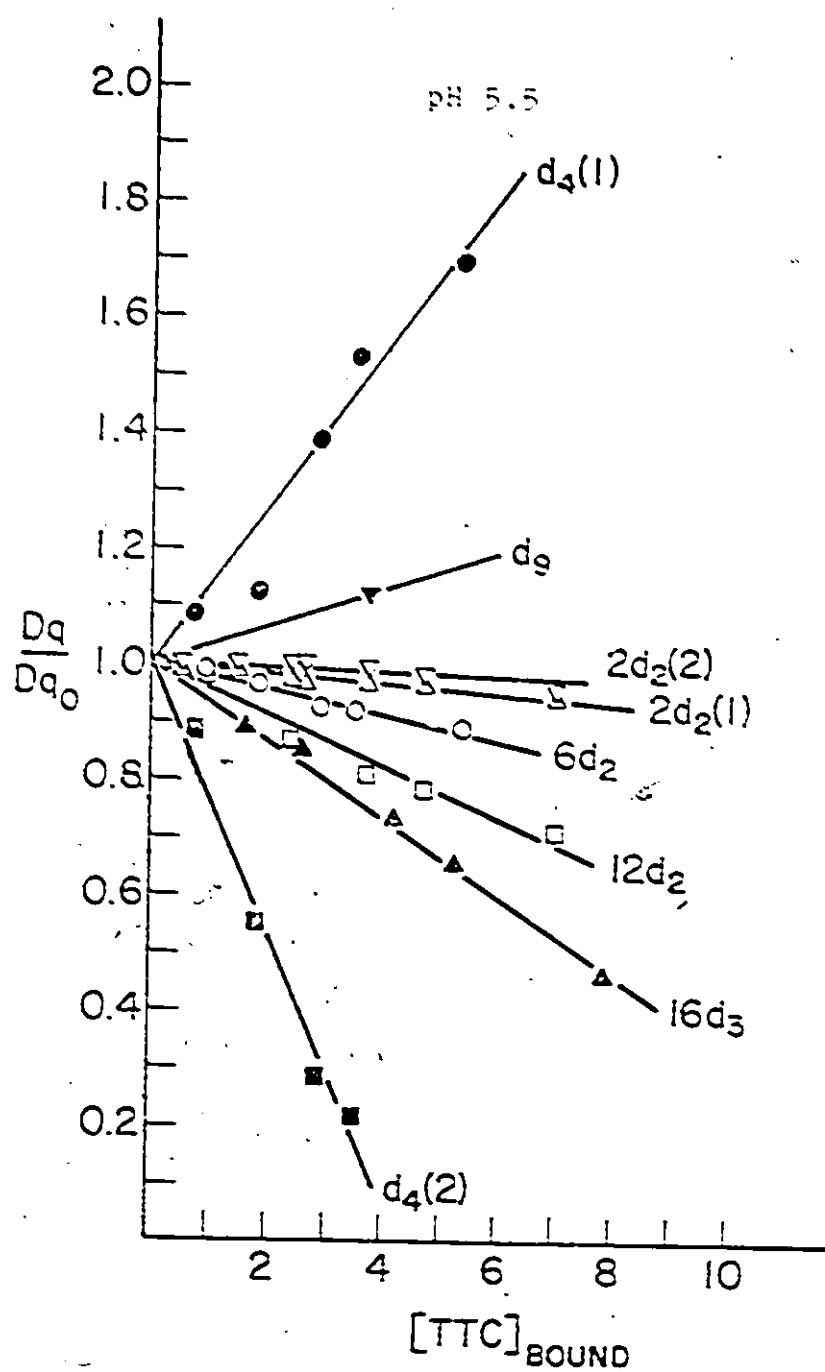


Fig. 31. Graph of the ratios of quadrupole splittings (D/D_0) of deuterated PC dispersions as a function of the amount of TTC bound at pH 5.5 (each unit corresponds to 0.05 mole TTC/mole PC). Values calculated from data in Fig. 29.

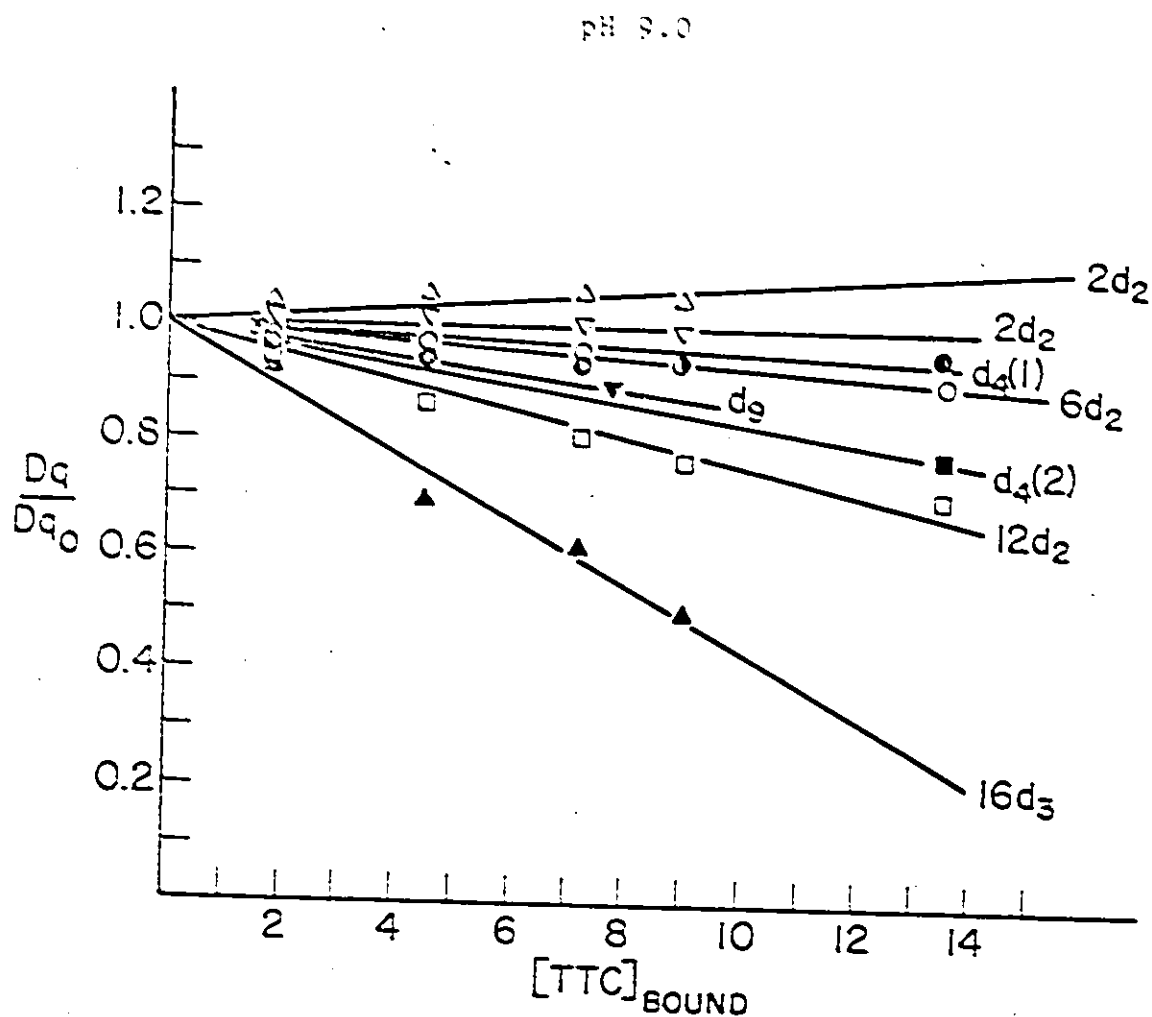


Fig. 32. Graph of the ratios of quadrupole splittings (D_q/D_{q_0}) of deuterated PC dispersions as a function of the amount of TTC bound at pH 9.0 (each unit corresponds to 0.05 mole TTC/mole PC). Values calculated from data in Fig. 30.

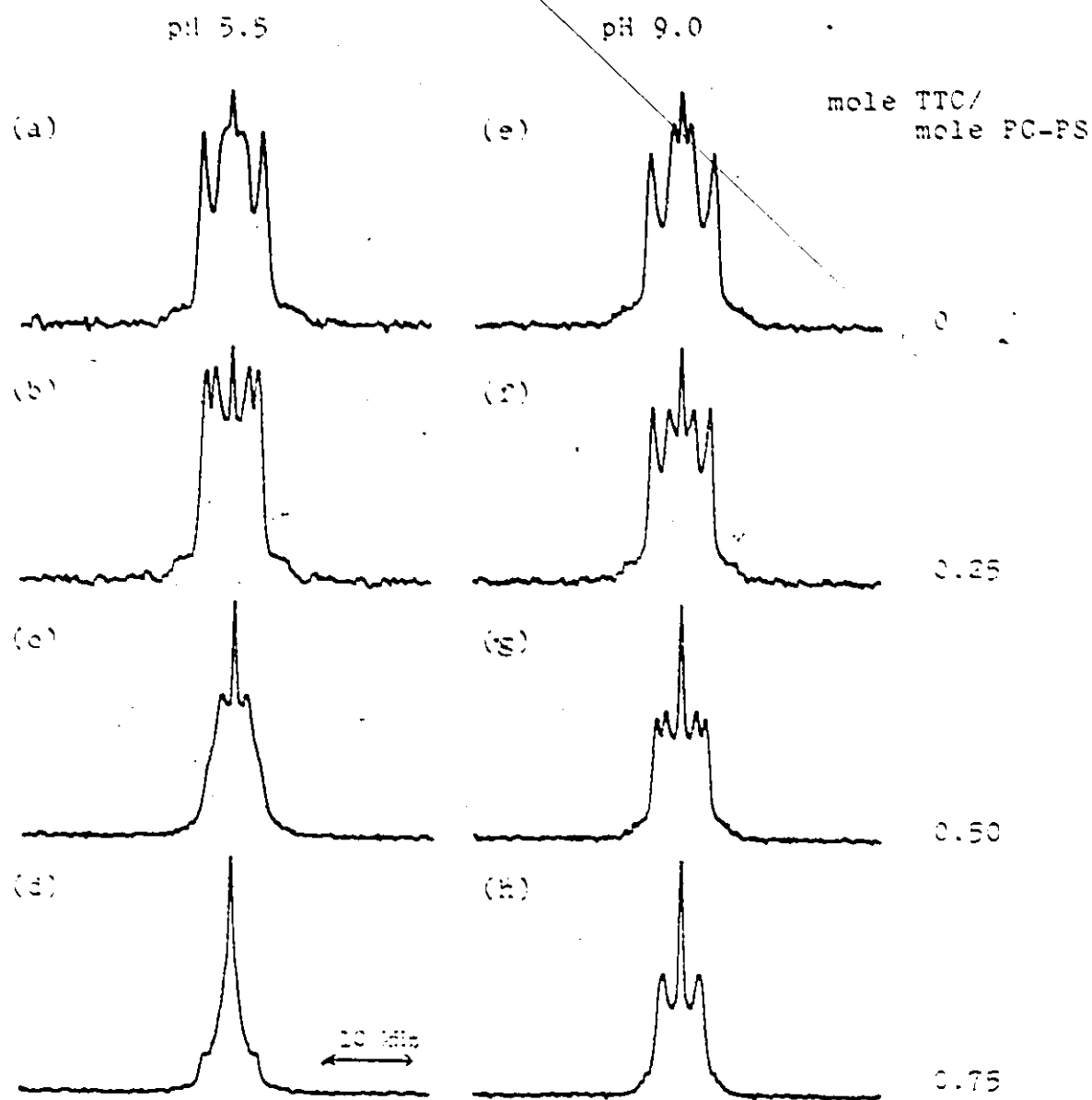


Fig. 33. ^2H NMR spectra (46.06 MHz) of PC- d_4 -PS 1:1 multilamellar dispersions at pH 5.5 (a-d) and 9.0 (e-h) containing a mole ratio TTC: phospholipid indicated to the right of the spectra. Samples were run at 30°C in BPC buffer. Spectra were acquired with the quadrupole echo technique ($\tau_1 = 60 \mu\text{s}$, $\tau_2 = 50 \mu\text{s}$) with 2048 data points, a 500 kHz spectral width, a 0.2 s recycle time, 12,000 scans and a 5 μs 90° pulse length.

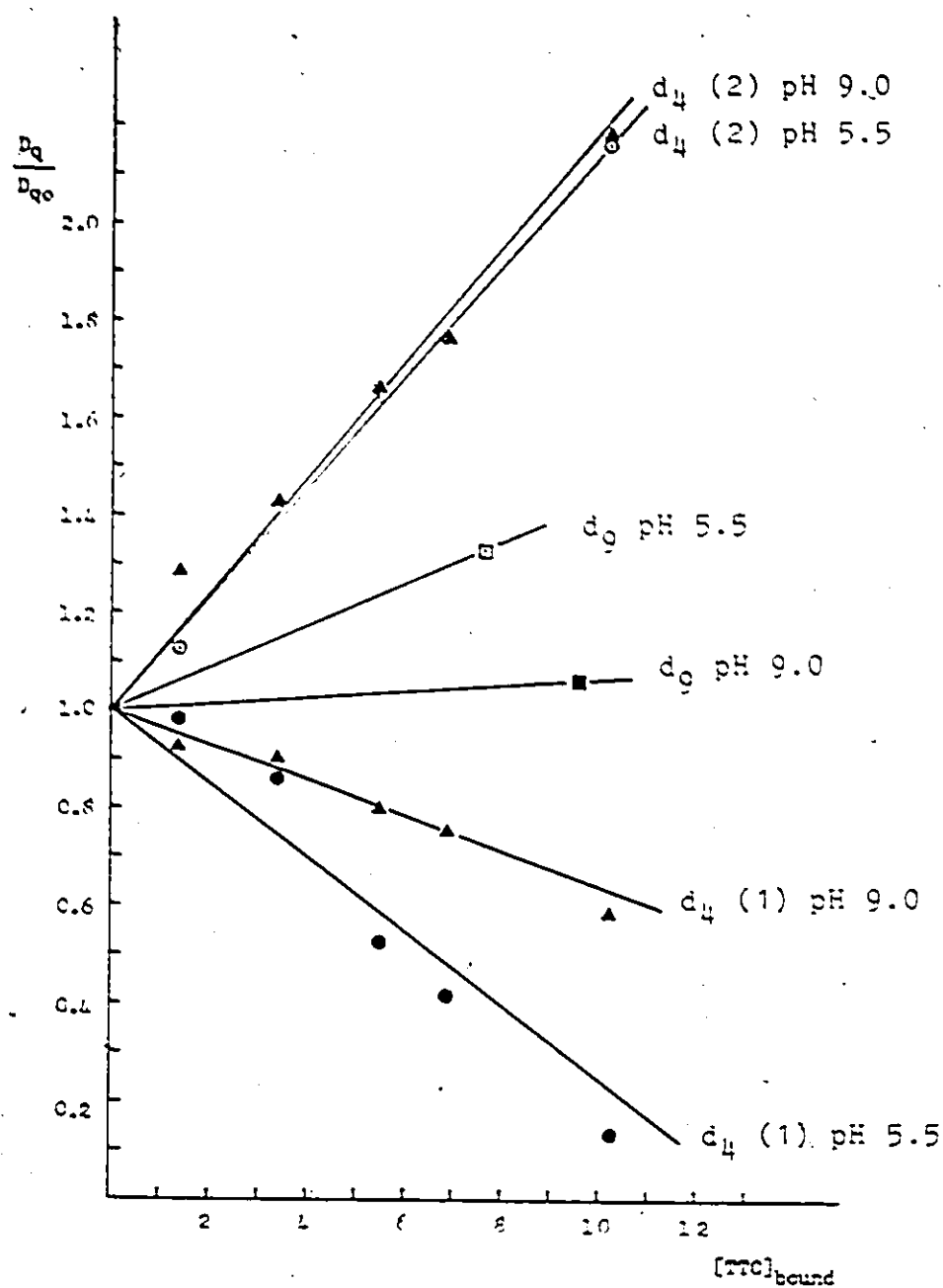


Fig. 34. Graph of the ratios of quadrupole splittings (D/D_0) of deuterated PC-PS 1:1 dispersions as a function of the amount of TTC bound (each unit corresponds to 0.05 mole TTC/mole PC).

D. ^{31}P NMR results

Addition of equimolar TTC to a PC multilamellar dispersion produced an increase of 8% in the $\Delta\sigma$ parameter of the ^{31}P NMR powder pattern at pH 5.5. No measurable change was found at pH 9.5 or when PRC was used. Fig. 35 shows the proton decoupled ^{31}P NMR spectra obtained for PC dispersions in the absence and presence of TTC at pH 5.5 and 9.5.

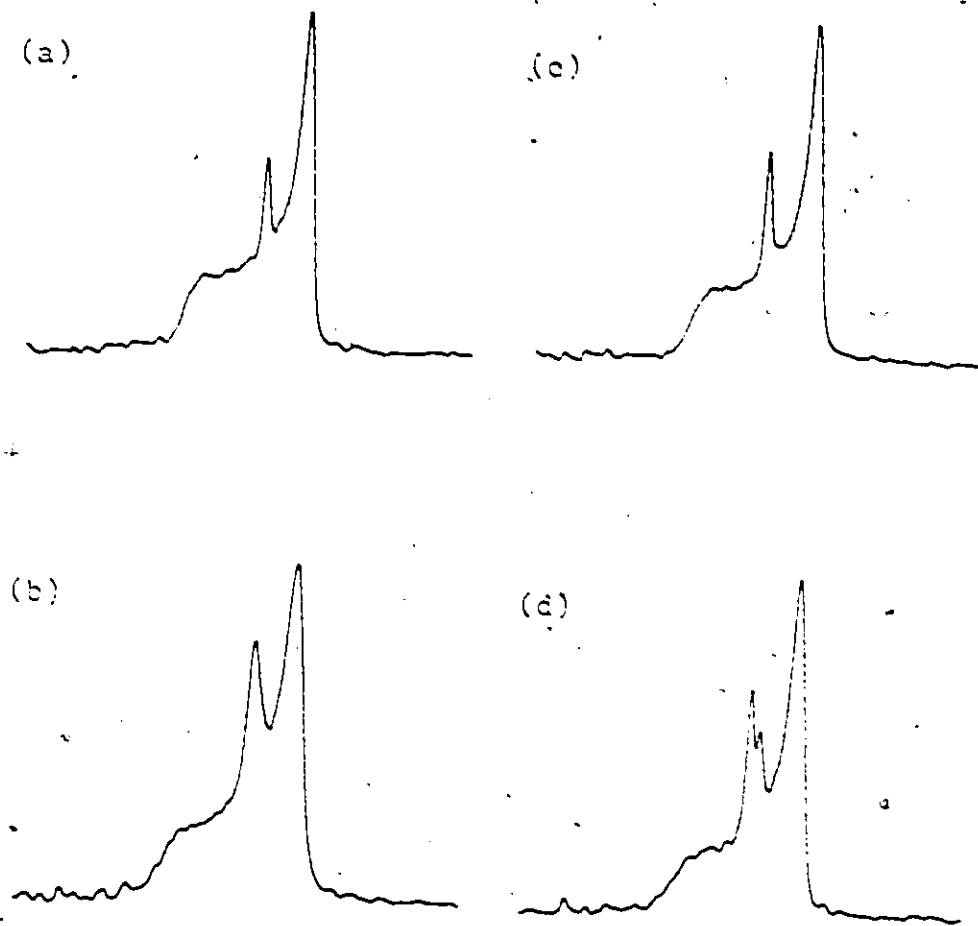


Fig. 35. Proton decoupled ^{31}P NMR (121.39 MHz) spectra of (a) 260 mM PC dispersions, pH 5.5, (b) 130 mM TTC in 260 mM PC dispersions, pH 5.5, (c) 173 mM PC dispersions, pH 9.0 and (d) 87 mM TTC in 173 mM PC dispersions pH 9.0. The narrow resonance is due to the phosphate ion of the BPC buffer. Spectra were acquired with 4096 data points, a 125 kHz spectral width, a 2 s recycle time, 2500 scans and a 4 μs 90° pulse length.

V. DISCUSSION

A. Calculated spectra in the presence of chemical exchange

Calculation of ^2H NMR spectra resulting from the exchange between a quadrupole splitting and a single resonance consists in the summation of an infinity of doublets affected by chemical exchange. The effect of exchange on each doublet depends on the difference in frequency between the components of the doublet and the resonance of the unbound species. In the same spectrum, the rate of exchange can be slow for some resonances, intermediate for others and fast for the remaining part of the spectrum.

The example given in Fig. 15 shows different possible lineshapes resulting from chemical exchange between a single resonance and a powder pattern. Slow exchange is illustrated in spectrum a; the two signals are superimposed. As the rate of exchange is increased, a broadening of the central resonance, caused by the exchange with the central components of the quadrupole splitting, is observed (spectrum b). As the central resonance broadens, the ratio of the heights is modified (spectra c and d) until the two signals merge (spectra

e and f). Increasing the rate of exchange yields separate peaks which become sharp when the rate of exchange is fast (spectra g-j). In Fig. 15, having an equal population of each site, the quadrupole splittings of the spectra obtained in the presence of fast exchange correspond to half the value of the original quadrupole splitting (1.5 kHz). This is in agreement with the value predicted by Eq. 18 for fast exchange.

The equations derived in Section II.B are a modification of the Gutowsky and Holm equations (80). No additional assumption was necessary in the derivation so that the relevance of the final equation is comparable to the case where both transverse relaxation times are equal (as in a doublet resulting from spin-spin coupling). The possibility of using different transverse relaxation times renders the equation more general and allows its application to powder pattern type of spectra.

B. Characterization of the system

(a) pK_a values

The pK_a values of the aromatic amino groups (Table 4) are the same as reported previously (95). In the case of the tertiary amino groups, other pK_a values (25) agree with those of Table 4 for PRC, but is larger for TTC. However, as given in Table 4b and as reported earlier (96), the pK_a value of TTC varies with anesthetic concentration. The pK_a value at 26 mM in Table 4b is in agreement with that reported for the same concentration (46).

(b) Partition coefficients

The determination by difference of the amount of anesthetic (Section I.C.a) bound to phospholipid multilamellar dispersions leads to some uncertainty in the estimated values of the partition coefficients, especially when the anesthetic concentration in one phase is small. However, the reproducibility of the results and the agreement with the NMR data (Fig. 21) suggest that the degree of accuracy is satisfactory.

(Table 5)
The present values for K_p can be compared to

those obtained in water: oleyl alcohol at pH 7.2 (25): PRC, 0.6; TTC, 80. These should be intermediate between those measured here at low and high pH. If we take into account the inaccuracy of the value for PRC at low pH, and the fact that different partitioning phases were used in the two studies, our values with PC or PC-PS dispersions are in reasonable agreement with these data. The partition coefficient for TTC in PC dispersions can also be compared to a value of 1405 obtained with DPFC at high pH (97). This value was, however, determined at higher temperature in the region of the phase transition. No data exists for conditions identical to those used in the following experiments.

The actual values for partition coefficients in PC-PS and PC-PI multilamellar dispersions are comparable to those obtained for PC multilamellar dispersions at pH 9.5 but are greater at pH 5.5. This is a clear indication that either an electrostatic interaction or a structural difference in head group causes an increase in binding of the local anesthetic to the phospholipid. The charge effect does not exist at pH 9.5 and thus, the partition coefficients measured are not affected by the presence of PS.

(c) Amount of charged and uncharged local anesthetic

The results in Table 6 indicate the amount of local anesthetic in each form inside and outside the membrane. It seems unlikely that 50% of PRC can be bound at pH 5.5 with 650 mM PC. This is certainly attributable to the inaccuracy of the partition coefficient resulting from the low amount bound.

It is interesting to note that in the membrane, the amount of TTC in the uncharged form at pH 5.5 represents 30% of the amount of anesthetic bound. As a consequence of the different partition coefficients of the charged and uncharged forms, the pK_a value of the local anesthetic is reduced inside the membrane and yields a substantial amount of both forms in the membrane. In view of the concentration dependence of the pK_a value for TTC, it is likely that the membrane concentration of the uncharged form will be lower than 30% of the total anesthetic bound.

(d) Fatty acid composition of phospholipids

Depending on the source, the fatty acid composition of a phospholipid class may vary from one preparation to the other. However, for a common source (e.g. egg

yolk PC), the proportions of each fatty acid should be in the same range. The results obtained with egg yolk PC and egg yolk PE (Table 8) are in reasonable agreement with published data (16, 99). The composition of the two fatty acid chains of egg yolk PC are quite different (99): the chain in position 1 is mostly saturated with more than 90% palmitic (16:0) and stearic (18:0) acids but the fatty acid chain in position 2 is mostly unsaturated with more than 90% unsaturated 18-carbon fatty acid chains. No data about the fatty acid composition of beef brain PS or wheat germ PI has been found in the literature. From our analysis (Table 8), beef brain PS is the most unsaturated among these four phospholipids.

(e) ESR experiments

The spectra of multilamellar lipid dispersions containing high concentrations of PC-5-SL display asymmetric resonances due mainly to spin exchange interaction (Fig. 18). The degree of asymmetry was evaluated by the c/d ratio given in Fig. 18. As the extent of spin-spin interaction decreases, this ratio tends to 1 (100). This would be the case if the spin-

labelled molecules became more dilute by incorporation of additional components into the membrane, or if the anesthetic caused a decrease in the lateral mobility (diffusion rate) of the lipid molecules (Eq. 3). The c/d ratio is seen to decrease upon addition of increasing amounts of TTC (Fig. 19) changing by 44% at pH 7.8 and by 15% at pH 5.5.

The results at pH 7.8 were taken as an indication that partitioning of anesthetic between membrane and aqueous phase is fast and that it equilibrates between the latter and all lipid bilayers of the multilamellar system in a time no longer than that required for mixing. The smaller effect at pH 5.5 seems to be explained by time-dependent experiments which showed that at this pH, complete equilibrium was attained only after a period of 3-5 hours (Section IV.B.f).

These data are not in agreement with an earlier conclusion (101) that TTC does not pass from the outer to the inner monolayer of unilamellar vesicles in an unbuffered medium. Since only a fraction of the lipid is located in the outermost monolayer of multilamellar vesicles (5-8%, ref. 102), a smaller effect would be expected if the anesthetic did not cross the outermost bilayer and hence were unavailable to subsequent bilayers.

(f) Hydrolysis of local anesthetic and DPPC

Calculations using reported rate constants for alkaline hydrolysis predicted that less than 2% of PRC or TTC would be hydrolyzed at pH 9.5 during a period of 36 hours at 35°C (103). These data confirm that local anesthetic hydrolysis is of minor importance and that it can be ignored for the interpretation of the spectra, which were usually recorded in less than 36 hours after sample preparation (Section IV.B.g).

The hydrolysis of DPPC at pH 9.0 and 47°C is negligible over a period of 10 hours (Section IV.B.g). This pH value was used to record spectra of specifically-deuterated DPPC dispersions, since the effect of lyso PC, produced by hydrolysis, might be severe on the ^2H NMR spectra. The hydrolysis of DPPC in water at 50°C has been reported to be less than 1% after a period of 9 days (94).

(g) Size of lipid and local anesthetic particles

The absence of change in light scattering measurements (Section IV.B.h) indicated that no change in the size of multilamellar PC-PS dispersions was occurring upon addition of local anesthetic. This was confirmed

by ^{31}P NMR experiments (Fig. 35) which showed that only a minor amount of isotropic phase, giving rise to the single resonance, existed in the presence of local anesthetic at high pH.

Light scattering measurements of the aggregation of local anesthetics in solution have also been reported (104). These data showed that PRC and TTC in water form only small aggregates of 2 and 4 molecules, respectively. In the presence of 0.4 M NaCl, more complex multiple equilibria exist between different aggregates. Another study showed that the critical micellar concentration of TTC was of the order of 70 mM (105), above the concentrations normally used in the NMR experiments. Thus, the presence of those aggregates or micelles can be neglected in the interpretation of the NMR results.

(h) Choice of optimal experimental conditions

Two pH values, 5.5 and 9.5, have been used throughout most of this work. At those pH values, the aromatic amino groups of the local anesthetics are completely uncharged because of their low pK_a values (Table 4). In water at pH 5.5, more than 99% of PRC and TTC are charged at their tertiary amino groups.

At pH 9.5, 99% of TTC and 76% of PRC are uncharged in aqueous solution. These two pH values were chosen because they allowed to study the effect of the charged^{us} and of the uncharged forms of the local anesthetic molecules. In the membrane, however, both the charged and uncharged forms exist at pH 5.5 but the charged form is predominant (Table 4):

The temperatures used for the NMR measurements were 30°C with egg PC and 47°C with DPPC multilamellar dispersions. At those temperatures, the phospholipids are in the liquid crystalline state (Table 1).

At pH 9.5, the problem of hydrolysis of the local anesthetic can be neglected at 30°C. When DPPC dispersions were used, experiments were performed at pH 9.0 and 47°C to avoid hydrolysis of the phospholipid and of the local anesthetic.

The ^2H NMR experiments require relatively large concentrations of local anesthetic. At high pH, these concentrations usually exceed the solubility of the local anesthetic in water (Table 7). However, in all cases where the anesthetic concentration has been decreased (factor of 10), for NMR experiments and determination of partition coefficients, no significant change in the results has been noted. It can be reasoned

that the local anesthetic precipitated at the beginning of the dispersion, the partitioning into the lipid membrane would lower the concentration of anesthetic in water which in turn would allow the dissolution of the precipitated anesthetic, and the process would continue until equilibrium was reached. Since the partition coefficients at pH 9.5 are high (Table 5), the amount of local anesthetic left in aqueous solution will be below its solubility.

C. Deuterated local anesthetic in PC dispersions

(a) Variation of lipid: water ratio: the three site model

The presence of a quadrupole splitting of constant magnitude upon dilution, and of a broad resonance whose linewidth decreases with addition of water (Section IV.C.a.ii), is indicative of two states for the bound anesthetic in slow exchange with one another. The local anesthetic can then be (1) free in water, (2) weakly-bound (probably near or at the bilayer surface), and (3) strongly-bound (at least partly intercalated between the fatty acid chains)(Fig. 36).

A fast exchange between site (1) and (2) gives rise to the dependence of the linewidth on the lipid: water ratio (Fig. 22), whereas site (3) is in slow exchange with both sites (1) and (2) and is responsible for the quadrupole splitting (constant magnitude but decreased intensity upon dilution, Fig. 21).

The population in each site depends on the nature of the local anesthetic, and on the conditions (pH, lipid: water ratio) of the experiment. At pH 5.5, both a constant quadrupole splitting and a resonance of

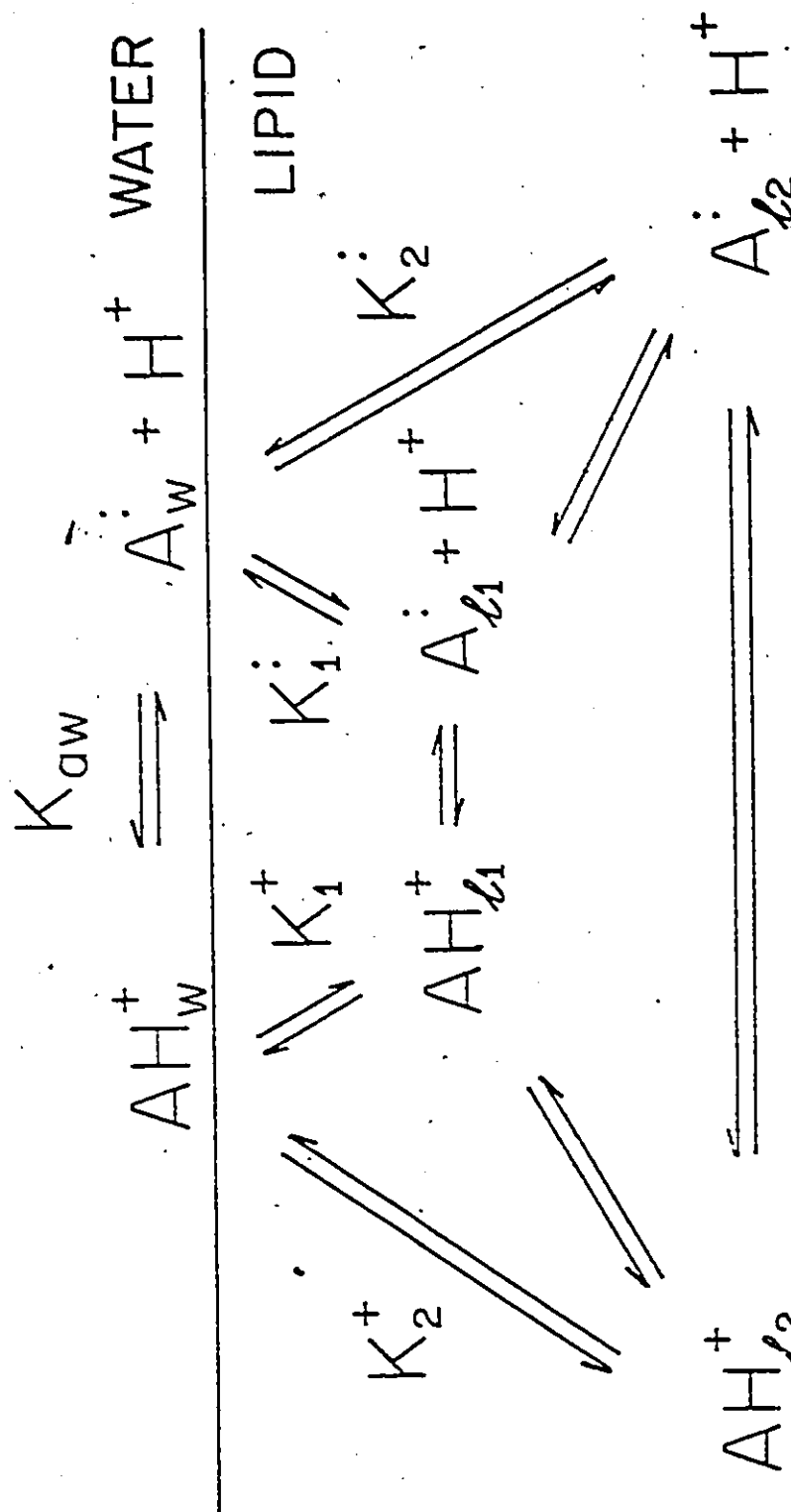


Fig. 36. Scheme of possible equilibria for the local anesthetic in both charged (AH^+) and uncharged (A) forms between water (w) and lipid (l) phases. The subscripts 1 and 2 refer to the weakly- and strongly-bound anesthetic, respectively.

variable width are observed with TTC, indicating that the three sites are populated in substantial amounts (Fig. 21). At pH 9.5, where most of the TTC is partitioned into the lipid, the same type of spectrum is observed, but only a very weak central resonance is present. In that case, the populations in sites (1) and (2) are very low, and a quantitative study of the effect of the lipid: water ratio on the width of the central line was not performed. For PRC at pH 5.5, only sites (1) and (2) are possibly populated (viscosity effect) and no quadrupole splitting was observed under the experimental conditions. At pH 9.5, similarly to TTC at pH 5.5, the three sites were occupied.

The large linewidths of the anesthetic resonances in the presence of lipid cannot be ascribed to an increase in viscosity of the bulk solution; the latter was only 1.38 times larger in lipid dispersions (52 mM) than in water alone. This would lead to a linewidth in the presence of lipid of approximately 3-14 Hz, well below the observed values.

The carbon-13 and proton resonances of PRC and TTC (mainly those of the aromatic region) are broadened in the presence of phospholipid (101, 107-112). However, no direct evidence for a strongly-immobilized species

was seen. It is likely that the ^1H and ^{13}C resonances of this species were too broad to be detected by the high resolution NMR methods employed. The ^{13}C and ^1H NMR data are in agreement with our conclusion that a fraction of the membrane bound anesthetic is in fast exchange with its counterpart in the aqueous phase.

Different membrane binding sites have been proposed for another anesthetic, halothane (113). Using ^{19}F NMR, three different signals were observed for that fluorinated general anesthetic in DPPC multilamellar dispersions. It was proposed that halothane could occupy four sites, (A) the bulk aqueous phase, (A') the aqueous phase-phospholipid interface, (B) the hydrocarbon chain near the phospholipid head group and (C) the hydrocarbon chain closer to the terminal methyl group. Basically, these proposals are in agreement with the conclusions derived for PRC and TTC. In the latter case, sites (B) and (C) might apply to the strongly-bound species at low and high pH.

(b) Partition coefficient for the weakly-bound species

Equation (43) was applied to the data in Fig. 22 for TTC in PC multilamellar dispersions at pH 5.5.

The value obtained for K_p by a least square fit of the data was 5.2. Thus the experimentally-determined partition coefficient (Table 5) is the sum of those for the weakly- and strongly-bound species. The calculated linewidth for the weakly-bound species was 660 Hz (probably a small quadrupole splitting). Making use of the data in Table 6 and the results obtained above ($\Delta\nu_q = 660$ Hz), it was possible to calculate that the amount of weakly-bound TTC was ca 23% of the total TTC bound at pH 5.5. Although it is not possible to evaluate exactly the amount of TTC weakly-bound from the ^2H NMR spectra, these values seem in general agreement with the observed spectra.

Comparison of the spectra in Fig. 21a and 21b show a significant difference in the linewidth of the single resonance. For the freeze-thawed sample (Fig. 21b), the spectra were recorded with a high power probe where the linewidth values are unreliable because of the absence of spinning and the design of the probe. It is to be noted that the ratio of the areas of the quadrupole splittings and the single resonances for the spectra in Fig. 21a and 21b are different suggesting that the freeze-thawing process changes the structure of the lipid bilayer. A reduction in D_q value (~400

Hz) seems to confirm that hypothesis.

(c) Membrane location of the strongly-bound species

None of the deuterated PRC species gave observable splittings at pH 5.5 (Table 9). At pH 9.5, D_Q for PRC- d_2 was much smaller than that for TTC- d_2 ; the D_Q value for PRC- d_4 cannot be directly compared to that for TTC- d_6 , since the deuterons are in a methylene group in the former compound. Nevertheless, a D_Q value of 1520 Hz is small for a C^2H_2 group; as a comparison, the D_Q value for the ultimate methylene group of stearic acid in PC dispersions was 8480 Hz (114):

The results for PRC could be indicative of faster exchange between anesthetic strongly-bound to the membrane and that free in water than in the TTC case. This is suggested by the smaller partition coefficients for PRC, which could result from a faster dissociation from the membrane. However, changes of the water: lipid ratio did not alter the value of D_Q obtained for PRC- d_4 at pH 9.5. Thus, the smaller quadrupole splitting for PRC must be ascribed to binding to a site of relatively low order. In addition, the small value

of D_Q for PRC- d_2 could also be due to the lack of an aliphatic chain bound to the aromatic amine. This, in fact, renders the molecule much less hydrophobic, and much less constrained in the benzenoid nucleus, than TTC, and would account for the weaker binding to the membrane.

A molecular order parameter, S_{mol} , can be calculated for TTC- d_2 from the values of quadrupole splittings (Eq. 16), assuming the axis of molecular ordering to pass through the 1,4 positions of the benzene ring: $S_{mol} = 0.73$ at pH 5.5 and 0.66 at pH 9.5. These values are higher than those obtained for the deuterons attached to the first ten carbon atoms of the PC acyl chains (69). In PC, $S_{mol} = 0.5$ for the first ten methylene groups, and decreases thereafter to 0.1 for the terminal methyl group (69). The increased values of S_{mol} for TTC over the highest S_{mol} seen for the PC acyl chains is probably at least partly due to the rigidity of the benzene ring. The order parameters, however, are lower than that observed for cholesterol in the same system ($S_{mol} = 0.85$, ref. 115). The fused ring region of cholesterol is known to intercalate between the first ten segments of the fatty acyl chains of the phospholipid (116).

In spite of the similarity of D_Q values for TTC-d₂, at low and high pH, the values of the quadrupole splittings for TTC-d₆ and TTC-d₃ vary on going from low to high pH (TTC-d₃ does not yield a quadrupole splitting at low pH, Fig. 20). This suggests that the membrane location of the strongly-bound site is pH-dependent. At low pH, the anesthetic should be closer to the water-membrane interface in view of its positively charged dimethylamino group. The large values of S_{mol} for TTC-d₂ suggests that the benzene ring of both the low and high pH strongly-bound forms of the anesthetic is located no deeper than carbon-10 of the fatty acyl chains in the bilayer. The low values of D_Q for TTC-d₆ and TTC-d₃ are mainly a result of labeling the terminal positions, for which the order is intrinsically low (due to rotational and lateral motion of the methyl groups). In addition, the bulky benzenoid ring of the anesthetic will force apart the fatty acyl chains of the phospholipids; as this ring has a larger molecular area than the attached alkyl chain, the terminal methyl group in TTC-d₃ could be expected to find itself in a region of decreased molecular order. A disordering effect of TTC is observed for the methyl group of stearic acid-18,18,18-d₃ intercalated in PC.

multibilayers at pH 5.5. Upon addition of TTC, the D_q value decreased from 2280 Hz to 1360 Hz.

The large values of S_{mol} for TTC- d_2 also indicate that the long molecular axis of the anesthetic is approximately parallel to that of the phospholipid acyl chains. This is consistent with calculations which lead to the conclusion that the anesthetic molecule is essentially linear (117).

The behaviour of TTC- d_6 and TTC- d_3 gives some insight into the location of the strongly-bound anesthetic at low and high pH. Taking the data for 260 mM lipid, it is seen that at pH 5.5, TTC- d_6 yields a quadrupole splitting of 1840 Hz, whereas TTC- d_3 manifests only a single resonance of width 200 Hz. At pH 9.5, both compounds show quadrupole splittings, 780 Hz and 800 Hz, respectively. Since the substituted ammonium group of TTC resembles that of PC at pH 5.5, it is plausible that these moieties be located in a similar region of the bilayer. This would place the benzenoid ring in the region of the glycerol moieties of the phospholipid molecules, causing an increased separation of the phospholipid acyl chains, and would have a quadrupole splitting smaller than expected (the splitting for the methyl group of lauric acid-12,12,12- d_3 in egg

PC dispersions is 4.56 kHz at 30°C, compared to 22.2 kHz for stearic acid-12,12-d₂ in the same system, ref. 58). Above the pK_a of TTC, however, one would expect the neutral substituted amino group to be able to drop deeper into the bilayer, where the perturbation by the benzenoid nucleus can be more easily accommodated by gauche-trans isomerization (kink or jog formation) of the phospholipid acyl chains, leading to a lesser increase in intermolecular separation, and hence to an observable D_q for TTC-d₃. A deeper position of the anesthetic molecule in the PC bilayer may also be consistent with a reduction in the TTC-d₆ D_q value.

These data with deuterated local anesthetic cannot provide an unequivocal solution to the location of the local anesthetic molecule in the lipid bilayer. A more complete picture of the interaction will be derived from work using specifically-deuterated phospholipids which will be discussed in Section V.E.

(d) Nature of the weakly- and strongly-bound anesthetic

One question that arises is whether the weakly- and strongly- bound species are chemically identical. Since the anesthetic can be present in solution in both

the charged and uncharged forms, one might speculate that the weakly- and strongly-bound sites correspond to the former and the latter, respectively. At pH 5.5, the charged form of TTC is present at a ratio 100:1 with respect to the uncharged form in the aqueous phase. However, preliminary work from this laboratory (118) indicates that the pK_a value of both PRC and TTC in the membrane are significantly decreased relative to those in solution. In addition, fluorescence experiments (119) are indicative of a change in the pK_a of PRC of about one unit due to location in the membrane.

The concentrations of each form in the membrane are calculated assuming the equilibria shown in Fig. 36. AH^+ and A are the charged and uncharged forms of the anesthetic and the subscripts w and i refer to the water and lipid phases, respectively, and 1 and 2 to the weakly- and strongly-bound species, respectively. K^+ and K^- characterize the partitioning of the charged and uncharged forms and K_{aw} is the equilibrium constant for the protonation of the local anesthetic in water^a. The different pK_a values for the anesthetic in the

^aThe standard notation for the partition coefficient is K_p . In order to simplify the presentation of all the p terms in the scheme of Fig. 36, the p subscript is deleted.

lipid and in water are a direct result of the different partition coefficients for the charged and uncharged forms.

The partitioning of the anesthetic into the membrane can be described by:

$$[AH^+] + [A:] = K^+ [AH_W^+] + K^- [A_W:] \quad (58)$$

Experimental determination of the partition coefficient at two pH values should enable one to calculate both K^+ and K^- (Section II.A), the total partition coefficients for the charged and uncharged species ($K^+ = K_1^+ + K_2^+$; ($K^- = K_1^- + K_2^-$)).

Using data for pH 5.5 and 9.5, calculated values for the partition coefficients of the charged species of PRC and TTC were 2.0 and 15, respectively, and for the uncharged species 59 and 660, respectively. Thus, for charged TTC, $K^+ = 15$, $K_1^+ = 5.2$ and therefore, $K_2^+ = 9.8$.

Preliminary results (118), however, suggest that the partition coefficient for the charged species varies as a function of pH (as a result of the alteration of the surface potential generated at the membrane by the charged form of the anesthetic). This would not allow the use of Eq. 58 as described. Nevertheless, the data

of reference (118) indicate that pH 9.5 is sufficiently far from the pK_a of the membrane-bound anesthetic to allow accurate calculation of the value of K^+ , which can be used in Eq. 59 at pH 5.5 to find K^+ .

Using this approximation, the percentages of total anesthetic present in the charged and uncharged forms in the membrane were calculated (Table 6). At low pH, less than one third of the bound anesthetic is uncharged. This represents a range of 14 to 28% of the total anesthetic for the lipid concentrations used in our experiment. These concentrations would not account for the amount of drug giving rise to quadrupole splittings in the 2H NMR spectra. Therefore, the subspectrum probably originates from at least two strongly-bound species, one uncharged and one charged. The values of D_Q at low pH for TTC- d_6 , TTC- d_2 and TTC- d_3 are different from those at high pH (Table 9), indicating that the contribution to spectra of the strongly-bound charged species predominates at low pH. However, under no conditions were the spectra indicative of separate splittings for the strongly-bound charged and uncharged forms.

(e) Simulated spectra

Due to the similarity between some spectra in Fig. 15 at intermediate rates of exchange between two sites and our observed spectra, simulations of the spectra of Fig. 21a were performed (Fig. 23). The spectrum (e) of Fig. 23 satisfactorily corresponds to the spectrum (e) of Fig. 21a. However, staying in the same range of residence times but changing the ratios of the line to the quadrupole splitting to account for the variation in the percentage of anesthetic bound, the spectra change dramatically and do not correspond to the spectra of Fig. 21a (a-d). In fact, the range of residence times where intermediate exchange is occurring is extremely limited and the shape of the spectra changes rapidly with a very small difference in the rate of exchange. Thus, the results of Fig. 23 are a confirmation that the spectra in Fig. 21a are not a result of an intermediate exchange between two sites.

The spectra of Fig. 24 were calculated assuming the three-site model proposed earlier (Section IV.C.a). The amount of strongly-bound species was obtained from the spectra of Fig. 21a (integration of the quadrupole

splitting). The amount of weakly-bound species was calculated from the partition coefficient determined by linewidth variation as well as its linewidth (660 Hz, Section IV.C.b). The results are in good agreement with the spectra of Fig. 21a and they support the three-site model derived from that experiment. Small differences in the spectra may be attributable to the inaccuracy of the experimentally-determined parameters (linewidth of the weakly-bound species, amount of strongly-bound species....)

D. Deuterated local anesthetics in PC-PS dispersions

(a) Exchanging sites present

All ^2H NMR spectra of specifically-deuterated local anesthetics showing a quadrupole splitting, also display a single broadened resonance in the center. Upon addition of phospholipid PC-PS 1:1, the width of the central resonance increases (Fig. 26) whereas the width of the quadrupole splitting remains unchanged. These observations are indicative of two binding sites to the PC-PS membrane, one in slow exchange (strongly-bound) and one in fast exchange (weakly-bound) with the local anesthetic in solution. This behaviour is identical to that in PC dispersions and is adequately represented by the equilibria shown in Fig. 36.

The linewidth of the central resonance is plotted as a function of the lipid concentration in Fig. 26 for several anesthetic and pH conditions. These data can be fitted to straight lines in all cases. The values of the slopes for these curves qualitatively follow the partition coefficient values. In particular, TTC and PRC have very similar slopes at pH 5.5 and 7.4 in agreement with the fact

that not much change in the K_p value is expected below the pK_a value of the anesthetic. An increase in slope by a factor of 6 is measured as compared to a factor of 8 in partition coefficients between low and high pH for PRC. This similarity between slopes and K_p values may suggest that the weakly-bound site is an intermediate site between the water and lipid phases. The anesthetic molecule in the weakly-bound site would occupy a peripheral region, possibly in the ionic double layer of the membrane.

In view of the experimental difficulties, the linewidth data must be considered only qualitative. The quality of the equipment used to obtain those data may also be a source of problems. More reliable data are now being acquired, and their quantitative treatment will be the object of another study.

(b) Membrane location of the strongly-bound species

No quadrupole splitting could be observed for PRC at pH 5.5 (Table 10), but a small variation in linewidth (Fig. 26) was measured, indicating that the charged form of PRC binds weakly to the PC-PS membrane. At pH 9.5, a small quadrupole splitting for PRC- d_4 and

a broad resonance for PRC-d₂ were observed. The absence of a quadrupole splitting for PRC-d₂ suggests that the benzenoid nucleus of this anesthetic is located in a position of very low order. The deuteromethylenes on PRC-d₄ and the aromatic C-D groups on PRC-d₂ being both in a penultimate position, the existence of a quadrupole splitting for PRC-d₄ clearly shows that this end of the molecule is in a more restricted environment than the aromatic ring portion. The polarity of the aromatic amino group, benzenoid nucleus and ester linkage also seems greater than that of the remaining triethylamine-like end (uncharged form). The bulkiness of the benzenoid nucleus is another factor which could hinder the penetration of that part of the molecule into an environment of high order. Thus, because of structural differences between PRC and TTC, the polarities of the molecules seem inverted and so may be their positions in the phospholipid bilayer.

In its charged form, TTC-d₃ shows a broad resonance and TTC-d₆ displays a small quadrupole splitting; the converse is observed for the uncharged form. This result certainly indicates a change in the position of TTC. TTC-d₂ gives rise to large quadrupole splittings from which values of S_{mol} of 0.79 and 0.69 can be cal-

culated at low and high pH, respectively. The benzenoid nucleus is certainly located in the most ordered parts of the bilayer membrane i.e. the glycerol backbone and the first ten segments of the fatty acyl chains (69).

These results are very similar to those obtained in the absence of PS (Table 9), except in the case of TTC-d₆ at high pH where a quadrupole splitting smaller than that at low pH was observed. This discrepancy reinforces the proposal that a part of the TTC molecule is in the head group environment. The difference in the degree of saturation of the fatty acyl chains between PC and PS cannot give rise to important changes in order since both have an equivalent percentage of unsaturated fatty acyl chains; however, the head groups of PC and PS are structurally different and are likely to cause variations in order which will be reflected in the deuterated TTC spectra. Therefore, at pH 5.5, the dimethylamino part of TTC is likely to be in the head group region, the aromatic nucleus at the level of the glycerol backbone and the n-butyl chain of TTC between the fatty acyl chains of PC and PS. At pH 9.5, the molecule of TTC penetrates deeper in the first ten carbon segments of the fatty acyl chains but the exact level is impossible to determine from these data.

Whether the dimethylamino group of TTC remains in the head group region or whether a flip-flop of the molecule occurs at high pH could be resolved only by supplementary studies. On polarity grounds, the location of the more polar dimethylamino group in the polar head group region of the phospholipid appears more probable.

At pH 9.5, a second negative charge starts to appear on the PS molecule ($pK_a = 10$, ref. 20), which may result in an increased repulsion between the molecules and in a general disordering of the bilayer. Comparing the PC- d_9 (~1050 Hz) and the PC- d_9 -PS (~750 Hz) quadrupole splitting values at pH 9.5, the difference may be due to the disordering caused by the second charge on PS. The penetration of the TTC into the bilayer may thus be facilitated (small effect).

(c) Electrostatic vs hydrophobic interactions

At the two pH values chosen, the PC molecule is zwitterionic whereas the PS molecule is negatively charged. The pK_a value of the PS amino group is about 10 (20) and a certain quantity of a second negative charge is building up at pH 9.5 (~30%). Since, at low pH, the local anesthetic molecules are positively charged

speculations about an electrostatic interaction anesthetic-PS at this pH are justified.

A first indication for a charge interaction is given by a comparison of the partition coefficients in PC and in PC-PS dispersions (Table 5). The K_p values in PC-PS dispersions at low pH are four fold larger than in PC dispersions alone for both PRC and TTC. Such a difference is not measured at high pH, the K_p values being in the same range (Table 5). The increased partition coefficients, because they were observed only in the charged form, are probably not due to the different structures of the head group of the phospholipid but rather to an electrostatic interaction. The partition coefficients in the negatively charged PI dispersions show the same behaviour (Table 5).

At high pH, the local anesthetic molecule loses its proton and becomes neutral. Its binding in the uncharged form increases substantially (Table 6). The same phenomenon was noticed in zwitterionic PC dispersions. These observations clearly demonstrate that the charge interaction is not the predominant force involved in the binding at high pH and that the hydrophobic interactions are more important. Moreover, the large difference in the K_p values between TTC and PRC

is indicative of a substantial contribution of the hydrophobic interaction to the binding. Considering the non-polarity of the major part of the phospholipid, the hydrophobic interaction seems to constitute the driving force leading to the intercalation of the local anesthetic molecule into the phospholipid bilayer. The hydrophilic interactions, because they would be much favored in the aqueous medium, are unlikely to play an important role in this case. They do, however, affect the positioning of the molecule in the phospholipid environment: the polar part of the local anesthetic will prefer the polar part of the phospholipid.

These results are in agreement with ^1H NMR (108, 109) and ESR (120) studies performed with PS dispersions, which stated that both hydrophobic and electrostatic effects are responsible for the anesthetic-PS interaction, the relative importance of each one depending on the nature of the anesthetic. Other studies have shown an antagonism between TTC and Ca^{+2} (121, 122) but the conclusion that TTC is more active at low pH (121) is inconsistent with our partition coefficients and ^2H NMR measurements. A more complete discussion of the force involved will be given after localization of the TTC molecule in Section V.E.c).

E. Local anesthetics in deuterated PC dispersions

(a) Location of local anesthetic in the bilayer membrane

At pH 5.5, the quadrupole splittings of perdeuterated PC multilamellar dispersions are reduced upon addition of TTC (Fig. 26). The resolution of the peaks in the DPPC- d_{62} pattern is lost when TTC is added and the intensity of the pattern grows towards the middle as a result of a decrease in its quadrupole splitting components. The use of specifically-deuterated DPPC molecules demonstrated that the D_q values of every position of the fatty acyl chain are decreased upon addition of TTC (Fig. 29). The relative effect of TTC on the D_q values increases with the carbon position in the fatty acyl chain; this is illustrated by plotting D_q/D_{q0} as a function of the concentration of TTC bound calculated from the partition coefficients (Fig. 31 and slopes in Fig. 37). These results seem to confirm a position of the TTC molecule in the head group region or at the level of the first positions of the fatty acyl chains (Section IV.C.c, Fig. 38). Such a position would separate the phospholipid molecules and create extra-available space all along the chain. The effect

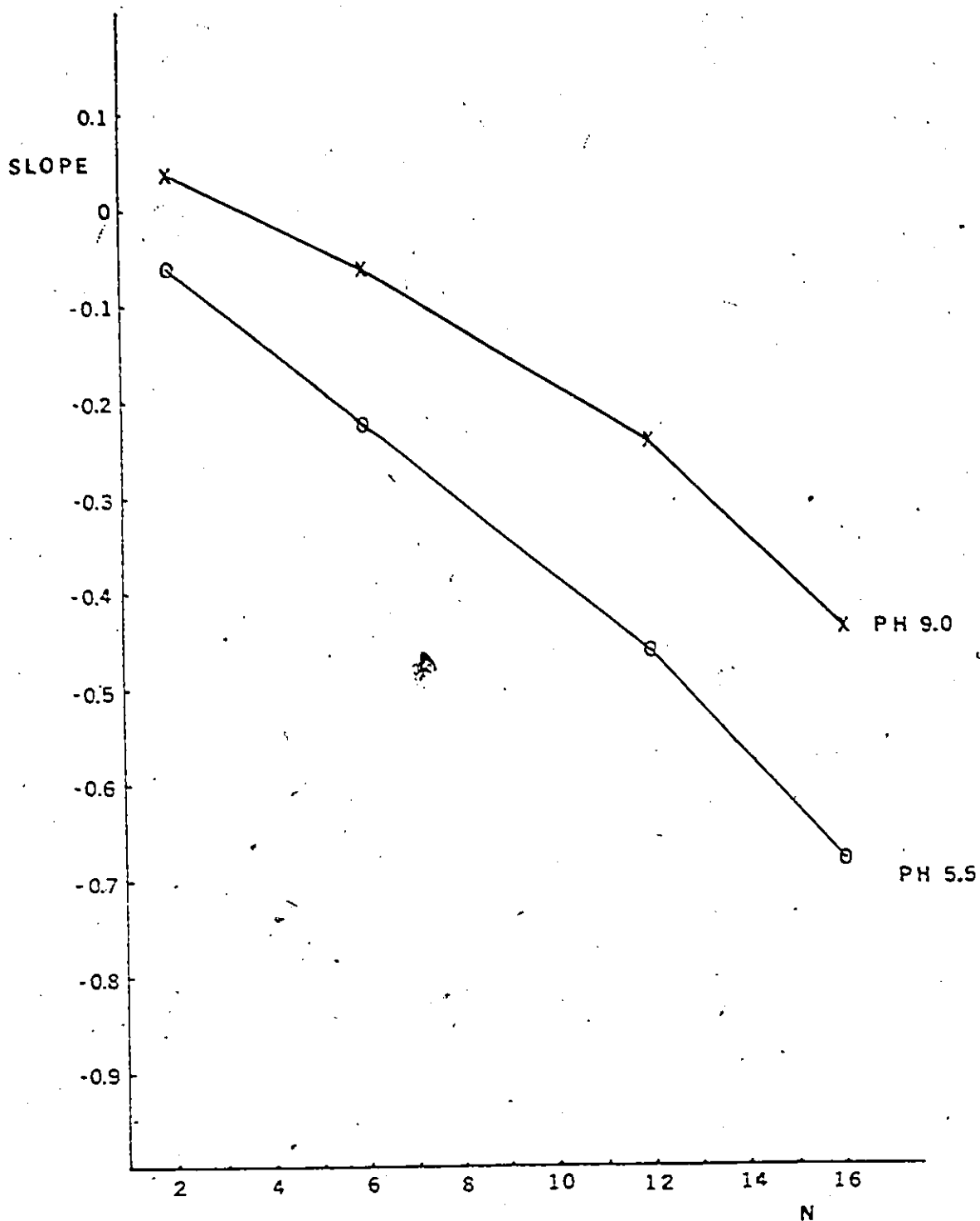


Fig. 37. Slope of the curves D/D_0 vs $[TTC]$ bound (Figs. 31 and 32) as a function of carbon position in the fatty acyl chain of the phospholipid at pH 5.5 and 9.0.

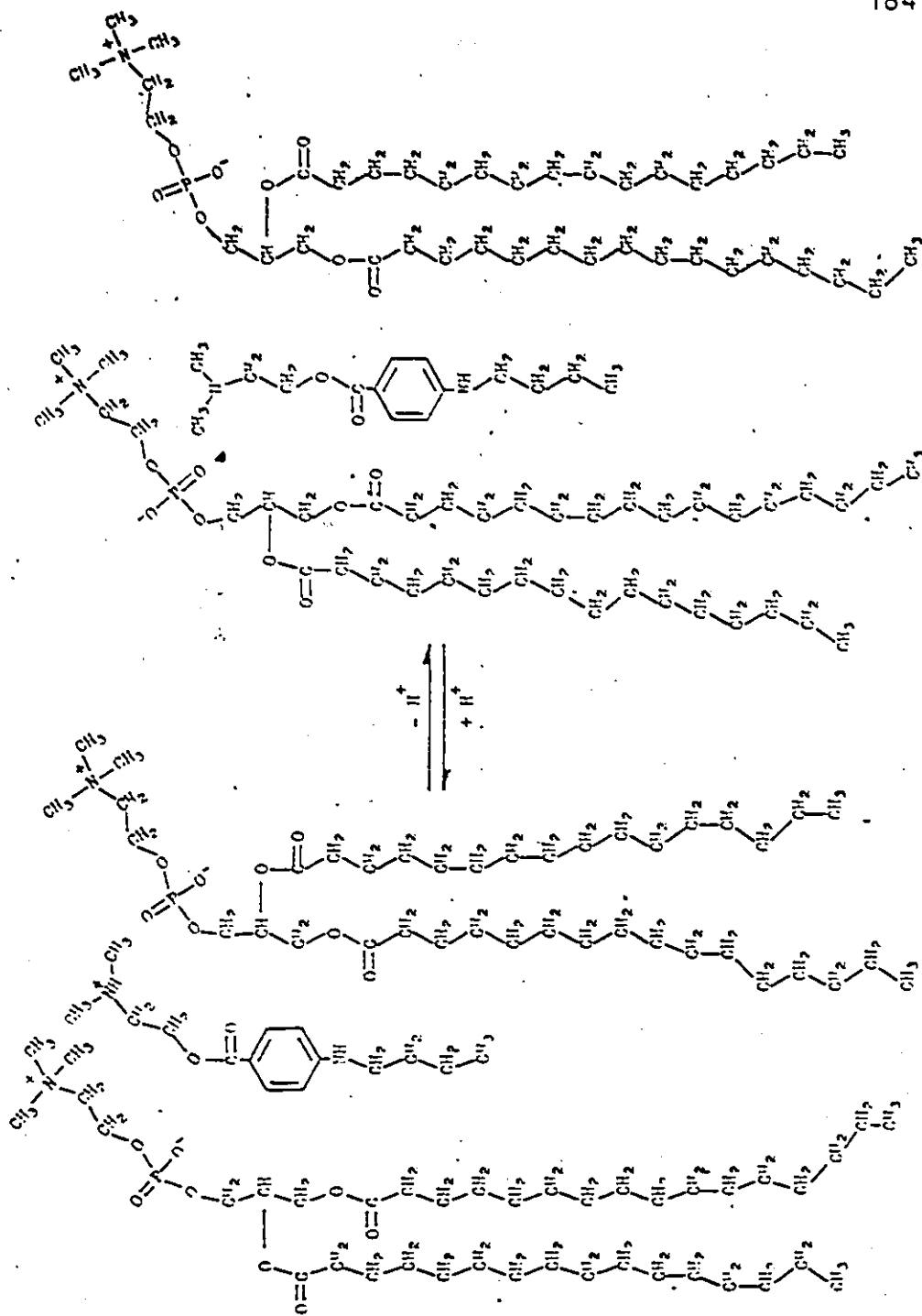


Fig. 38. Model for the interaction of local anesthetic TTC in the PC lamellar dispersion. The positively charged TTC at low pH (left) remains mostly at the phospholipid head group level and the uncharged TTC at high pH is intercalated partly in the head group and partly in the fatty acyl chains of the phospholipid.

on the order would thus increase progressively with the position of the carbon in the fatty acyl chains (analogous to the oscillation of a pendulum).

Addition of TTC to head group deuterated PC dispersions yielded unexpected results. Both the PC- d_9 and the methylene group of PC- d_{11} linked to the nitrogen showed an increase in D_Q value but the methylene group linked to the phosphate group in PC- d_{11} showed a decrease in D_Q value upon addition of TTC at pH 5.5 (Figs. 28 and 29). A part of the TTC molecule must be in contact with the PC head group and that part is most likely the charged dimethylamino portion of the TTC molecule which can be neutralized by the counterions of the buffer. The diminution of the quadrupole splitting of the methylene linked to the PC phosphate group results probably from the extra space below the two ammonium groups of PC and TTC in contact. Similarly to the effect on the fatty acyl chains, an increased level of conformational freedom leads to a reduction in the D_Q value. Another possibility is that the TTC molecule would fix the head group in a more rigid position which would make an angle close to the magic angle with the main director axis (Section I.C.c.i) and then reduce the quadrupole splitting.

This proposal seems unlikely in view of the opposite effect observed on the neighbouring carbon segment.

At pH 9.0, the same phenomenon is observed for the chain-deuterated DPPC molecules in the presence of local anesthetic i.e. reduction in quadrupole splittings except that the effect is smaller for all positions of the fatty acyl chain (Figs. 30, 32 and 37). For the carbon-2 of the fatty acyl chain, a very small increase in the quadrupole splitting is seen, suggesting that the bulky aromatic ring of TTC might be at that level (Fig. 38). The reduced effect on the other carbon segments relative to the results at pH 5.5 are in agreement with such a location.

The results for the three deuterated head group positions of PC reinforce the proposed location because they manifest a reduction in their D_Q values. The separation at that level by the local anesthetic in a deeper position increases the probability of trans-gauche isomerization in the head group (disordering) and yields a lower S_{CD} value. It is likely, then, that the TTC molecule penetrates in a position where its aromatic nucleus will be in the neighbourhood of the first positions of the fatty acyl chains.

- The PC-PS system at pH 5.5 has a behaviour iden-

tical to that of the PC system (Section IV.C.c). The charged TTC molecule must also be located at the level of the head group of the phospholipids. At pH 9.0, both the PC- d_0 and the first methylene of the head group show an increase in quadrupole splitting whereas the second methylene of the head group shows the opposite effect (Fig. 33). These changes are in the same direction but much smaller than at pH 5.5. Although these data could be explained by a position comparable to that of TTC at low pH, it seems more likely, considering the difference in the magnitude of the perturbation and the deuterated anesthetic data (Section IV.C.b) that such is not the case and that a different conformation of the PS head group, caused by the TTC molecule, is more plausible. Thus, the position would be the same as in PC multilamellar dispersions.

It has also been shown by ^2H NMR of specifically-deuterated DMPC dispersions that benzyl alcohol disorders the fatty acyl chains (123). It appears that this local anesthetic molecule would interact at the same level as PRC or TTC i.e. in the head group region of the phospholipid.

An ESR study has shown a complex effect of local anesthetics on multilayers of ox brain white matter

lipids when a cholestane probe is used (96). Above the pK_a value of the local anesthetic, a disordering effect was observed, in agreement with our data. Below the pK_a value of the local anesthetics and at low cholesterol concentration, however, an ordering effect was found. In no case has an ordering effect on the acyl chains been observed by 2H NMR at low pH. The anesthetics have been shown to have a disordering effect on PC-cholesterol vesicles at sufficiently high anesthetic concentration using ESR spin label fatty acid probes at pH 7.2 (57). This effect is in agreement with our 2H NMR data. The discrepancies between NMR and ESR may be attributable to the bulkiness of the spin label probes which has an effect on the fatty acyl chains of the phospholipids as reported earlier (124, 125).

(b) Correlation between deuterated phospholipid and deuterated local anesthetic results

The D_q value of TTC- d_2 decreases slightly when pH is increased (Table 9). If the benzenoid moiety of the TTC molecule moves from the glycerol backbone region at low pH to the fatty acyl chains region at high pH, there should be a decrease in the order on the TTC aro-

matic nucleus (69, 126). The quadrupole splitting of TTC-d₆ at low pH is consistent with its position at the head group level, since PC-d₉ also displays a small quadrupole splitting. This D_Q value decreases at high pH, when the two methyl groups of TTC would be near or below the phosphate groups, the phospholipid head groups being more separated than usual because of the effect of the aromatic nucleus of TTC. The TTC-d₃ spectrum shows no quadrupole splitting at low pH; the effect of TTC at the glycerol backbone level must be disordering enough to annihilate the quadrupole splitting of this terminal methyl group.

At high pH, however, the effect of the aromatic nucleus is not as important as at low pH, because it can be accommodated by the gauche-trans isomerizations occurring in the fatty acyl chains. In this case, TTC-d₃ gives rise to a small quadrupole splitting (Table 9).

The ²H NMR signals of specifically-deuterated TTC in PC-PS dispersions resemble those observed in dispersions of PC alone (Table 10). The same change in location can be postulated. For TTC-d₆ at high pH no quadrupole splitting could be seen under any condition as compared to a small quadrupole splitting in

the corresponding PC case. This result may be interpreted as a confirmation of the location of the dimethylamino group in the head group region of the phospholipid, the D_q difference being due to the structure of the PS head group (shorter and conformationally different from PC).

The similarity of the TTC-d₂ D_q values in PC and PC-PS practically excludes any major difference in location between the two phospholipid environments. Small differences in D_q values can also be attributed to the fatty acid composition of PS relative to PC (Table 8).

(c) Consideration of specific forces involved

The main force responsible for the position of TTC at low pH given in Fig. 38 is the solvation of the positively charged dimethylamino group in a highly polar water medium. In fact, a charged species is usually insoluble in an apolar solvent. The positive charge of TTC is surrounded by anions of the buffer and by phosphate anions of the phospholipid, which can act as counterions. Considering information that suggests that the head group of the phospholipid would lie parallel to the bilayer surface (128), the positive charge

of TTC might also be almost in contact with the negatively charged phosphate groups. In the arrangement of Fig. 38, the other parts of the TTC molecule are in their best environment i.e. the hydrophilic part of the local anesthetic is surrounded by the hydrophilic part of the phospholipid and the n-butyl chain of TTC is intercalated between the apolar fatty acyl chains (hydrophobic interaction). Stacking interaction (dipole-induced dipole) may be regarded as possible between a polarizable bond (C-O or C=O) and the aromatic ring of the local anesthetic. Such interactions have been demonstrated for other biological molecules both in solution (128) and in the solid state (129). Hydrogen bonds involving the aromatic amino group of TTC and the carbonyl group of the phospholipid seem unlikely due to steric hindrance caused by the bulkiness of the aromatic nucleus.

At high pH, the loss of the charged group results in a deeper penetration of the TTC molecule in the phospholipid bilayer. The position given in Fig. 38 enhances the hydrophobic interaction between the n-butyl chain and the fatty acyl chains relative to the low pH situation. A stacking interaction between the carbonyl group (dipole) of the phospholipid and the ben-

zenoid nucleus (induced dipole) of TTC appears highly probable; the rest of the molecule remains surrounded by a polar environment.

On the basis of these well-known interactions, the location of TTC derived from ^2H NMR at low and high pH seems completely justified. No other position could accommodate both the hydrophilic and the hydrophobic part of the local anesthetic molecule.

(d) Effect of TTC on the dimensions of the phospholipid bilayer

Deuterium order parameters can be used to calculate the effective length L of a hydrocarbon chain with n segments (130):

$$\langle L \rangle = 1.25 \frac{n}{2} - \sum_{i=1}^n S_{CD}^i \quad (59)$$

This equation has been applied to the deuterated phospholipid results. The length of the hydrocarbon chain segments 2-16 in the DPPC bilayer decreased from 12.7 Å to 12.2 Å upon incorporation of 0.1 mole TTC per mole of DPPC at low pH. At high pH, this length passed from 12.6 Å to 12.3 Å when the same amount of TTC was added.

In terms of surface area per lipid molecule, that represents a variation from $63.4 \text{ \AA}^2$ to $66.0 \text{ \AA}^2$ at pH 5.5 and from $63.7 \text{ \AA}^2$ to $65.4 \text{ \AA}^2$ at pH 9.0. The charged species has a greater disordering effect and perturbs more the dimensions of the phospholipid than does the uncharged species.

For local anesthesia, the expansion of membrane area ranges between 2-4% and the concentration of anesthetic bound is of the order of 40 mmoles/kg membrane (24). For the same concentration of anesthetic bound, TTC would produce an increase in area of the phospholipid of 1-1.5%, the remaining expansion being due to the local anesthetic itself.

These data are in agreement with the conclusions of a ^2H NMR study using benzyl alcohol (123) but not with black lipid membrane capacitance and conductance measurements which have shown an increase in membrane thickness (12 $\text{\AA}$) using the same local anesthetic (131). The disordering effect on the phospholipid membrane caused by the anesthetic intercalation is an explanation for the reported decreases in the phospholipid phase transition temperatures (38). The anesthetic effect is opposite to the cholesterol effect which has shown an increase in membrane thickness and ordering (69).

F. Effect of local anesthetic on the PC phosphate group

A change in chemical shift anisotropy $\Delta\sigma$ in the ^{31}P NMR spectrum may be due to a change in order but may also originate from a modification of the ^{31}P chemical shift tensor without relation with the order (Section I.C.d). An increase in $\Delta\sigma$ was observed for TTC at low pH but no variation occurred with PRC or TTC at high pH or with PRC at low pH in PC dispersions (Section IV.D). Since a change in the chemical shift tensor would most likely result from a strong interaction such as electrostatic binding and since the ^2H NMR results suggest that the local anesthetic molecule simply intercalates without important electrostatic binding to the phosphate (Section V.C.c), the spectra can probably be interpreted in terms of order. An increase in order is detected only at low pH for TTC in agreement with the position of the aromatic nucleus determined by ^2H NMR and illustrated in Fig. 38. The latter results exclude the interpretation of the TTC effect in terms of a simple phosphate-ammonium electrostatic interaction proposed elsewhere (101). The location of TTC in PC dispersions at high pH given in Fig. 38 is consistent with the negligible effect observed in the ^{31}P NMR spectra (Fig. 35). The absence of par-

titutioning for PRC at low pH explains the lack of effect in that case while a peripheral and possibly inverted position for PRC would account for the lack of change in the ^{31}P NMR spectrum at high pH.

The single resonance observed on the ^{31}P NMR powder pattern before addition of local anesthetic arises from the phosphate ions contained in the BPC buffer. The increase in intensity of that single resonance at low pH and the appearance of a second peak at high pH are indicative of the formation of an isotropic phase (possibly vesicles) in minor amount. No important structural change is observed, unlike that observed with the other local anesthetics, dibucaine and chlorpromazine, which induced a transformation from bilayer to hexagonal phase in cardiolipin dispersions (132). Dibucaine also reversed the action of Ca^{+2} by transforming PS-PE 20:80 dispersions from the hexagonal to the bilayer phase (133).

VI. CONCLUSION

A. Summary

The techniques of ^2H and ^{31}P NMR have allowed the localization of the local anesthetic molecules PRC and TTC in model membranes consisting of PC and PC-PS 1:1 multilamellar dispersions. The binding of the charged and uncharged forms is different and is dominated by hydrophobic forces and solvation requirements. The PRC molecule, which binds only in its uncharged form, intercalates at the head group level and its aromatic part seems to be directed towards water. TTC binds in both the charged and uncharged forms and has its dimethylamino group closer to water. In its uncharged form, TTC penetrates deeper into the bilayer, partly between the fatty acyl chains and partly between the head group. These positions are relevant to the strongly-bound species but there exists also in all cases, a weakly-bound species whose location is at the lipid-water interface.

A theoretical treatment of the effect of chemical exchange has also been developed and its application to our conditions allowed an adequate simulation of the spectra.

B. Comparison with earlier models

On the basis of force-area measurements with monooctadecyl phosphate monolayers (134), PRC has been reported to bind stoichiometrically to the lipid. The area expansion produced by PRC was consistent with an horizontal position of the local anesthetic molecule in the head group region which would separate the molecules of octadecyl phosphate by a length corresponding to the length of the PRC molecule. Another study, using phospholipids and lipids from nerve and muscle (95), concluded that local anesthetics form 1:2 molar complexes probably by ion-ion and ion-induced dipole type of binding at the phosphate groups of the lipid. A model was drawn for TTC in the charged form where the two amino groups of the TTC molecule would interact with the phosphate groups of the phospholipid; the molecule would lie horizontally at the membrane surface but bent in a way that its n-butyl chain is oriented parallel to the fatty acyl chains. It is difficult to compare the PRC model with ours because a different lipid was used. However, considering the flexibility of the head group, the PRC molecule is unlikely to separate the PC or PC-PS molecules apart to a distance corresponding to the PRC length. Moreover, the absence

of effect of PRC on the ^{31}P NMR spectra of PC dispersions suggests that the local anesthetic-phosphate interaction is not strong. Our data are indicative of an interaction at the head group level but also of a parallel intercalation between the head groups of the phospholipid (Section V.D.b). The TTC-phospholipid interaction described above is completely inconsistent with our conclusions except for the fact that the TTC molecule is located in the head group region of the phospholipid.

^1H and ^{13}C NMR studies (101, 107-109) have all suggested a strong electrostatic interaction between the dimethylamino group of TTC and the phospholipid PO_4^- group. ^2H NMR data at pH 5.5 demonstrate that this is not the case but that the dimethylamino group of TTC is in contact with water. If the phospholipid head group lies parallel to the bilayer surface, the positively charged amino group of TTC might be surrounded by a phosphate environment, with no strong link to the immediately neighbouring molecules.



C. Significance of the results for the mechanism of local anesthesia

At the present time, there exists no consensus about the mechanism of action of local anesthetics. Theories about a lipid action and about a protein action are both defended but without definitive proof (Section I.A.c). In view of the strong binding of local anesthetics to phospholipids, theories based on an effect at the protein level cannot exclude a step involving an interaction with the lipid phase. Since no protein has been used in our experiments, it is not possible to support a definite mechanism from our results. The main contribution of this work is that the local anesthetic-phospholipid interaction has been characterized in a better way than in any earlier study, mostly because of the utility of the ^2H NMR technique. The existence of two binding sites and the localization of the strongly-bound site had never been demonstrated previously.

Supposing that a protein is responsible for the phenomenon of anesthesia, the lipid phase may serve as a channel through which the local anesthetic would come into contact with the protein molecule. As proposed,

earlier (135), the local anesthetic molecule might disorder the boundary lipid layer surrounding the protein and thus, perturb the conductivity process. In fact, a number of possibilities have been suggested; the definitive elucidation of them will come when more complex systems are investigated by NMR or similar physical techniques. Our results characterize the first part of a possibly complicated mechanism.

D. Future work

This local anesthetic project should be developed in several directions in the future. As mentioned earlier, a quantitative treatment of the partition coefficients and the relative peak areas in the ^2H NMR spectra using the Gouy-Chapman theory is in progress and will be reported later. Another project might be concerned with the use of cross-polarization techniques between the phospholipid and the local anesthetic in order to verify the locations derived from ^2H NMR. Eventually, the use of more complex membrane systems including proteins might lead to the complete elucidation of the mechanism of anesthesia...

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RESUME

Le mécanisme d'action des anesthésiques locaux sur les membranes des cellules nerveuses, bien qu'il ait été étudié par de nombreuses techniques, n'a pas été élucidé de façon définitive. Ce travail présente une étude de l'interaction des anesthésiques locaux, procaine (PRC) et tétracaine (TTC), avec des membranes modèles consistant de dispersions multilamellaires des phospholipides phosphatidylcholine (PC) et phosphatidylserine (PS) par résonance magnétique nucléaire (RMN) du deutérium et du phosphore-31. Ces techniques ont l'avantage de permettre l'observation d'une molécule dans un milieu où les mouvements moléculaires sont restreints, comme c'est le cas dans les systèmes biologiques.

Des molécules deutérées de procaine (PRC-d₂ et PRC-d₄) et de tétracaine (TTC-d₂, TTC-d₃ et TTC-d₆) ont été synthétisées. La phosphatidylcholine a été deutérée dans les chaînes d'acide gras (DPPC-x-d₂ et DPPC-16'-d₃) et dans le groupe de tête (PC-d₉ et PC-d₄). Après une caractérisation du système choisi (coefficients de partition, pK_a, solubilité, etc), les conditions optimales d'observation par RMN ont été déterminées. Les anes-

thésiques locaux ont été étudiés dans leur forme positivement chargée (pH 5.5) et dans leur forme non-chargée (pH 9.5) à des températures où les phospholipides se trouvaient sous forme liquide-cristalline. *

Les résultats suggèrent que la molécule d'anesthésique est liée de deux façons, faiblement et fortement i.e. en échange rapide et en échange lent avec le milieu aqueux environnant. Ces deux sites sont déduits du fait que deux types de résonance sont observés dans le spectre RMN, un spectre de poudre (écart quadrupolaire) correspondant à l'espèce fortement liée partiellement immobilisée et une résonance simple mais élargie dont la largeur de raie dépend de la quantité faiblement liée au lipide. La quantité d'anesthésique dans chaque site lié et dans l'eau dépend des conditions expérimentales: rapport eau: lipide, coefficient de partition, température. Un traitement quantitatif des coefficients de partition a permis de déterminer que les deux formes de l'anesthésique, chargée et non-chargée, sont fortement liées au lipide.

Les spectres RMN du deutérium d'anesthésiques locaux marqués en présence de dispersions de PC permettent d'affirmer que la position de l'anesthésique dans la membrane est différente aux deux valeurs de pH. Le grand

écart quadrupolaire observé avec TTC-d₂ à haut et à bas pH indique un haut degré de restriction i.e. le groupement aromatique se trouve dans une partie très ordonnée de la membrane (partie glycérol ou chaînes d'acides gras). Les écarts quadrupolaires des groupes méthyliques TTC-d₃ et TTC-d₆, varient de façon contraire sous augmentation du pH, ce qui indique un changement de position de la molécule dans la bicouche lipidique. Procaine ne se lie que faiblement à bas pH mais à haut pH, des écarts quadrupolaires relativement petits sont observés pour PRC-d₂ et PRC-d₄. La molécule n'est probablement liée que de façon périphérique au groupe de tête de PC.

7 L'emploi d'un mélange de PC et de PS pour la formation de dispersions multilamellaires démontre une liaison accrue à bas pH qui est attribuable à l'attraction de la charge négative de PS pour la charge positive de TTC. Les résultats de RMN pour TTC sont très semblables à ceux obtenus avec PC. Aucun écart quadrupolaire n'est observé pour PRC-d₂ à haut pH, contrairement à un faible écart avec PC, ce qui suggère que la partie aromatique de la molécule serait la plus rapprochée de la phase aqueuse. En effet, cette portion de la molécule est plus polaire que la partie contenant l'amine tertiaire quand celle-ci est non-chargée.

L'addition de TTC à des dispersions de PC spécifiquement deutéré dans les chaînes d'acide gras provoqua une diminution de l'ordre dans ces chaînes à bas et à haut pH, à l'exception de la position 2 de la chaîne à haut pH. L'augmentation de l'ordre dans le groupe de tête de PC pour les groupes méthyles et le groupe méthylène lié au groupement phosphate, indique une forte interaction à ce niveau. Dans sa forme chargée, la molécule de TTC s'intercale de façon parallèle au groupe de tête du phospholipide. La partie aromatique est alors située au niveau du groupement glycérol et la chaîne n-butyle pénètre au milieu des premiers carbones de la chaîne d'acide gras. A haut pH, on n'observe qu'une réduction légère des trois écarts quadrupolaires des groupes de tête. La molécule pénètre donc un peu plus profondément dans la bicouche lipidique qu'à pH 5.5 et le noyau aromatique est dans la région des premiers méthylènes de la chaîne hydrocarbonée. Les résultats des phospholipides deutérés sont en accord avec les données sur les anesthésiques deutérés et avec les spectres de phosphore-31 où un effet n'est observé qu'à pH 5.5 avec TTC.

Finalement, des équations ont aussi été dérivées pour calculer l'effet de l'échange sur les spectres RMN du deutérium et ont été appliqués avec succès aux spectres des anesthésiques locaux.