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NMR-STUDIES ON THE MOLECULAR BASIS OF DRUG-INDUCED PHOSPHOLIPIDOSIS—II. INTERACTION BETWEEN SEVERAL AMPHIPHILIC DRUGS AND PHOSPHOLIPIDS*

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Abstract—NMR binding measurements have been performed in order to analyze the molecular mechanism underlying the drug-induced phospholipidosis. The dependency of the T_2 relaxation rates of the various spin systems of the drug molecules on structure, type of lipid, concentrations of interacting species, and ionic strength has been evaluated. Increasing lipophilicity is correlated with an increase in binding for this type of amphiphilic drugs. The degree of signal broadening is determined by the ratio of drug/lipid concentration. Strong interaction occurs with phospholipids, like phosphatidylcholine or phosphatidylethanolamine, whereas less polar lipids, like diacylglycerole or digalactosyldiglyceride, show no interaction with the drugs. Cholesterol antagonizes the phospholipid/drug interaction.

Pharmacokinetic, biochemical, autoradiographic and ultrastructural studies revealed that certain amphiphilic drugs show an exceptionally high affinity for lipid-rich tissues which gives rise during chronic treatment to an impairment of lipid metabolism in several species including man [1, 5]. Due to distinct common physicochemical properties drugs with different pharmacological effects cause the same side action—namely the interference with phospholipid breakdown. The resulting accumulation of phospholipids leads to the formation of abnormal cytoplasmic inclusion bodies with multilamellated or crystalloid structure. From NMR binding studies with phosphatidylcholine and chlorphentermine it can be argued that in the mechanism of this side action a strong complexation between phospholipids and amphiphilic drugs is involved [2]. In order to ascertain that the proposed model for this type of side action is generally applicable, the NMR binding measurements have been extended to several other amphiphilic compounds with potential phospholipidosis-inducing properties. Additionally, the interaction of these drugs with other lipids under various experimental conditions has been studied.

MATERIALS AND METHODS

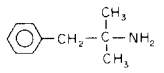
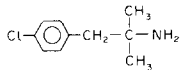
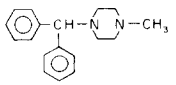
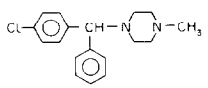
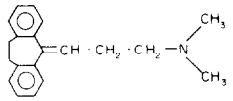
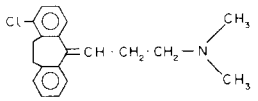
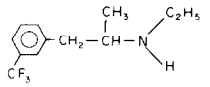
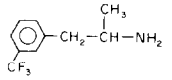
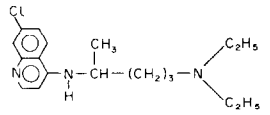
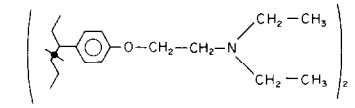
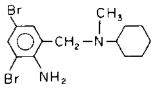
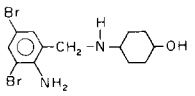
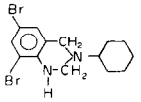
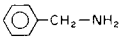
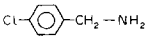
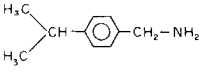
Two different types of phosphatidylcholine (PCh) were used in this study. Chromatographically purified egg yolk PCh (PCh_1) with a relatively high proportion of saturated hydrocarbon chains (16:0 36.2%, 18:0 11.6%, 18:1 36.4%, 18:2 9.4%, 20:4 3.7%, according to [12]) was kindly supplied by Dr. G. Blaschke (Dept. of Pharm. Chem., University of Bonn, Germany). Using $CHCl_3$, CH_3OH, H_2O (65:25:4) as solvent PCh_1 showed a single spot ($R_f = 0.2$) on Silica gel plates. A highly unsaturated PCh_2 from soya beans was generously supplied by Nattermann and Cie (Köln, Germany). The fatty acids consist of 85% of oleic, linoleic, and linolenic acids (16:0 12.5%, 18:0 2.6%, 18:1 9.0%, 18:2 70.1%, 18:3 5.8%). Using the same chromatographic system as mentioned above, PCh_2 is characterized by $R_f = 0.3$. Phosphatidylethanolamine (PE) and digalactosyldiglyceride (DGD) were obtained from Serva (Heidelberg, Germany). Diacyl glycerol was obtained from PCh_1 by enzymatic hydrolysis using phospholipase C (Boehringer, Tutzing, Germany, personal communication). The drugs and compounds used were chlorphentermine (Pre-Sate®, Warner–Chilcott, U.S.A.), phentermine (Mirapront®, Mack, Illertissen, Germany), chloroquine (Resochin®, Bayer, Germany), chlorcyclizine, cyclizine (Burroughs Wellcome, England), amitriptyline, 1-chloroamitriptyline (Hoffman-La Roche, Basel, Switzerland), fenfluramine (Ponderax®, Boehringer, Ingelheim, Germany), norfenfluramine (Benzon, Kopenhagen, Denmark), bromhexine (Bisolvon®) and its metabolites I and II (Thomae, Biberach, Germany), 4,4′-diethylaminoethoxyhexestrol (DEH) (Trimanyl®, Tosse, Hamburg, Germany), DEH was a gift from Dr. T. Shikata, (Tokyo, Japan), 4-isopropylbenzylamine, 4-chlorobenzylamine and benzylamine from Dr. G. Blaschke (Dept. of Pharm. Chem., University of Bonn, Germany). The compounds are listed in Table 1. All other reagents used were of analytical grade.

NMR spectra. All NMR spectra were recorded on a Varian HA 100 high resolution spectrometer at 22° with tetramethylsilane (TMS) as external standard. Most line-width measurements were made at a sweep width of 5 Hz/cm and at a sweep rate of 0.5 Hz/sec. Relaxation rates $1/T_2$ were calculated from at least three measurements using the equation:

$$1/T_{2\,\mathrm{obs}} = \pi \cdot \Delta \nu_{1/2}$$

* Part I: J. K. Seydel, O. Wassermann, *Naunyn-Schmiedeberg's Arch. Pharmac.* **279**, 207 (1973).

Table 1. Generic names and chemical structures of the compounds studied.

Generic name	Chemical structure
Phentermine	
Chlorphentermine	
Cyclizine	
Chlorcyclizine	
Amitriptyline	
1-Chloro-amitriptyline	
Fenfluramine	
Norfenfluramine	
Chloroquine	
4,4'-Diethylamino-ethoxy-hexestrol (DEH)	
Bromhexine	
Bromhexine metabolite I	
Bromhexine metabolite II	
Benzylamine	
4-Chloro-benzylamine	
4-Isopropyl-benzylamine	

where $\Delta\nu_{1/2}$ is the line width (mm) of the proton signals of the different groups shown in Table 2 at one-half maximum peak height. The broadening of the NMR signals is given in mm throughout the paper.

Controls. To ascertain the specificity of the observed line width broadening the following controls have been performed:

(1) Possible intermolecular associations between drug molecules. The influence on relaxation rates as a function of increasing drug concentration has been studied. Under the experimental conditions used in this study no change in relaxation rates occurred ($4–10 \times 10^{-2}$ M). The NMR spectra of the drugs studied did not show any alterations in line width at temperatures between 15 and 40°.

(2) Possible changes in relaxation rates due to alterations in viscosity. Since no changes of relaxation rates occurred in the presence of DGD and diacylglycerol, which should cause similar changes in viscosity as PCh_1 or PCh_2, the influence of viscosity on relaxation rates can be excluded.

For the different experimental procedures the following conditions were chosen:

(1). *Proton relaxation rates of the compounds studied as a function of different PCh concentrations.* Concentration range of PCh_1 and PCh_2 was 0–2.4 mg/ml D_2O phosphate buffer (0.01 M, pH 9.0, 7.4 or 6.0, resp.). Drug concentration was 4×10^{-2} M, the experiments reported were performed at pH 6.0 ± 0.1 after addition of the drugs. In pilot studies the concentration range of PCh was extended to 0–4.8 mg/ml and the drug concentration was doubled. To decrease the formation of myelin structures and to obtain a homogeneous distribution of PCh in the aqueous buffer system sonicated PCh solutions have been used (5 min 50 W at 4°, centrifuged at 5000 rpm for 5 min). The solutions were kept under N_2 at 4°, prior to the NMR measurement the solutions were allowed to equilibrate to 22°. The average mol. wt. of the resulting smaller micelles or vesicles is estimated to be 5×10^6 [3]. Unless otherwise mentioned the drugs were added to the PCh preparation shortly before the NMR measurements.

Influence of ionic strength on drug–PCh interaction was studied in the range of 0–5 mg NaCl/ml, pH was varied between 5 and 9.

(2). *Relaxation rates of the proton signals of norfenfluramine as a function of its concentrations at constant PCh concentration.* Concentration range of norfenfluramine at a PCh concentration of 2.4 mg/ml was $4–10 \times 10^{-2}$ M.

(3). *Relaxation rates of the proton signals of chlorphentermine in the presence of various lipids.* Studies were performed with PCh_1, PCh_2, PE, DGD, and diacylglycerol.

(4). *Influence of different aralkylamines on the NMR spectrum of PCh_2.* Sufficiently high concentrations (24 mg/ml) for this type of experiments could be obtained only by sonication of PCh_2 in D_2O. Concentration of the arylalkylamines was in the range of $2–12 \times 10^{-2}$ M.

(5). *Influence of cholesterol on the drug/PCh_2* interaction. 2.4 mg PCh_2/ml deuterated buffer and cholesterol (0–1.5 mg/ml deuterated buffer) were sonicated and chlorphentermine (4×10^{-2} M) was added.

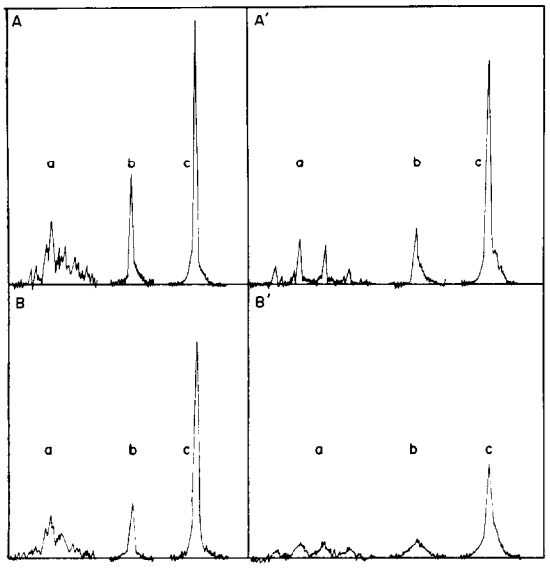


Fig. 1. NMR proton resonance signals of phentermine and chlorphentermine in the absence (A, A') and presence (B, B') of PCh_1 (2.4 mg/ml); a = aromatic, b = methylene, and c = methyl protons. Drug concentration, 4×10^{-2} M.

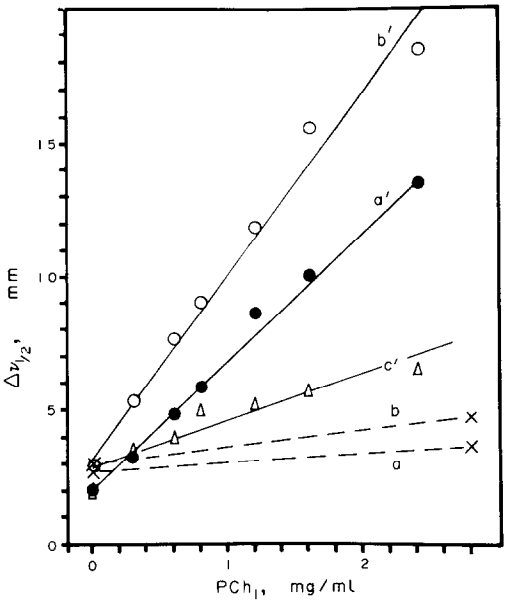


Fig. 2. Signal broadening ($\Delta\nu_{1/2}$, mm) of various spin systems of phentermine (a,b) and chlorphentermine (a',b',c') as a function of increasing PCh_1 concentrations (0–2.4 mg/ml). For the explanation of the symbols for the various spin systems see Fig. 1. a,b = phentermine, a',b',c' = chlorphentermine. For quantitative comparison see the slopes of the regression lines, equations (1a–1c).

RESULTS

(1). *Proton relaxation rates of the compounds studied as a function of different PCh concentrations.* The interaction between the drug molecule and PCh micelles was followed by studying the changes of the proton signals which are caused by changes in relaxation rates of the protons (it was assumed that under the experimental conditions the longitudinal relaxation time T_1 is equal T_2 and $1/T_2 \equiv \pi \Delta \nu_{1/2}$). Such changes in relaxation rates can also be produced by changes in temperature, viscosity or intermolecular associations between drug molecules at high concentrations. This could be excluded by appropriate controls (see Materials and Methods). The chosen drug/PCh concentration ratio allowed the observation of the drug proton signals without interference by PCh proton signals. For most of the compounds studied it was observed that the relaxation rates of the spin systems are characteristically altered in the presence of PCh. A typical example is depicted in Fig. 1 where the NMR spectra of phentermine and chlorphentermine are given in the absence (A, A') and presence (B, B') of PCh_1.

It is obvious from this graph that the presence causes a distinct broadening of the drug proton signals. Quantitatively, however, there is a remarkable difference in the degree of interaction if phentermine is compared to chlorphentermine. The large difference in the observed effect is even more astonishing if the close structural similarity is considered. It seems to indicate a specific interaction between the chlorphentermine molecule and PCh. If the observed changes in proton signals are due to an interaction between "binding sites" at PCh then the degree of interaction should depend on the concentration of these binding sites. The result of a corresponding study with increasing PCh_1 concentration at constant drug concentration is given in Fig. 2. The quantitative evaluation of the signals reveals that the various parts of the molecule are involved in the interaction with PCh to a different degree (Fig. 2, Table 2).

A slight interaction between PCh_1 and phentermine can only be observed at the highest PCh_1 concentration applied, whereas chlorphentermine shows a strong interaction (Fig. 2, Table 2). For the phenyl and methylene protons of chlorphentermine this interaction is linearly correlated to the increase of PCh_1 concentration. The per cent increase in line width broadening for these two spin systems is identical, despite the fact that the slopes of the regression lines in Fig. 2 are different. However, aromatic and methylene protons in the chlorphentermine molecule are more involved in the interaction than the methyl protons in the neighbourhood of the protonated hydrophilic amino group. For other drugs in Table 2, however, quantitative differences in the binding of aromatic and adjacent methylene protons have also been observed.

Both the striking difference in the signal broadening between phentermine and chlorphentermine and the observed changes in the relaxation rates for the various spin systems within one chlorphentermine molecule underline the specificity of the effect. In case of an unspecific interaction all protons of the same molecule should be affected to the same extent. It might be suggested that the hydrophobic bonding seems to be of essential importance for this type of interaction. Phentermine differs from chlorphentermine only by the lack of a chlorine atom attached to the aromatic ring system. This substitution changes the partition coefficient (P) (calculated using the fragmental constants of Nys and Rekker [4]) from log P,2.45 to log P,3.43.

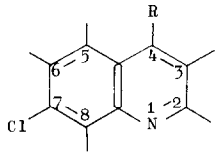
It was of interest to include other agents with pronounced hydrophobic properties of the ring systems which are known from *in vivo* experiments as inducers of a phospholipidosis [1, 5].

The compounds studied are summarized in Table 1. Due to poor solubility in water and/or complex spin systems, a quantitative analysis was possible only for the compounds listed below. In Table 2 the binding affinity of the various groups is expressed by the proportionality factor, m, of the equations (1a–7b)* which were obtained by correlating the increase of single broadening of the various spin systems (a, b, c, d) to the PCh concentrations.

	$y = (1/T_2)$ (mm)		$x_1 = PCh_1$ (mg/ml)			
	n	r	s	F	S (%)	
Chlorphentermine:						(eq.)
$y_a = 4.9\,x_1 + 2.01$ (26.6)	7	0.996	0.37	706	> 99.9	(1a)
$y_b = 6.7\,x_1 + 3.54$ (16.0)	7	0.99	0.41	258	> 99.9	(1b)
$y_c = 1.7\,x_1 + 2.89$ (5.62)	7	0.93	0.61	32	> 99.5	(1c)
Fenfluramine:						
$y_a = 1.6\,x_1 + 7.4$ (27.3)	7	0.997	0.119	747	> 99.9	(2a)
$y_b = 3.2\,x_1 + 4.47$ (17.2)	7	0.99	0.38	296	> 99.9	(2b)
$y_d = 2.2\,x_1 + 2.89$ (5.5)	7	0.93	0.81	30	> 99.5	(2d)
Norfenfluramine:						
$y_a = 1.4\,x_1 + 7.18$ (8.55)	6	0.98	0.33	73	> 99.5	(3a)
$y_b = 2.3\,x_1 + 3.3$ (29.2)	6	0.997	0.16	854	> 99.9	(3b)
$y_c = 1.8\,x_1 + 1.7$ (16.6)	6	0.99	0.21	274	> 99.9	(3c)
4-Chlorobenzylamine:						
$y_b = 1.3\,x_1 + 2.0$ (14.0)	6	0.987	0.19	196	> 99.9	(4b)
4-Isopropylbenzylamine:						
$y_a = 3.0\,x_1 + 0.93$ (12.4)	7	0.98	0.49	154	> 99.9	(5a)
$y_b = 5.7\,x_1 + 2.24$ (15.6)	7	0.99	0.37	244	> 99.9	(5b)
$y_d = 0.8\,x_1 + 1.65$ (9.6)	7	0.97	0.18	92	> 99.9	(5d)
Bromhexine, Metabolite I:						
$y_b = 4.8\,x_1 + 3.6$ (28.0)	5	0.998	0.22	794	> 99.9	(6b)
Chlorcyclizine:						
$y_b = 0.7\,x_1 + 3.97$ (6.54)	6	0.96	0.29	43	> 99.9	(7b)

For other drugs listed in Table 1 an interaction with PCh_1 can also be observed, but can only qualitatively be described. A representative example is given in Fig. 3a and 3b. Amitriptyline and its chlorinated derivative seem to interact with PCh_1 to a similar degree. Comparison of the spin systems of the N-methyl protons of both compounds in the absence of PCh_1 reveals that the introduction of the chlorine atom does not only alter the proton signals of the ring system but causes a pronounced change in molecular conformation. This is indicated by the observed splitting of the N-methyl-proton signals (d) of 1-chloroamitriptyline compared to the singlet obtained with amitriptyline. The expected stronger increase in interaction for the chlorinated derivative might therefore be inhibited by a conformational change.

Significant interaction with PCh_1 could be evaluated also for bromhexine, its metabolite II and for DEH. As a sign of specific interaction, again, the various spin systems are influenced to a different degree for these compounds. In chloroquine, for example, the ring protons 2 and 5 participate significantly more in binding than the protons 3, 6, and 8:



The difficulties which arise in structure–affinity relationship studies with such heterogenous compounds can be avoided if homologous series of compounds are used. This would facilitate quantitative structure affinity correlations to prove the importance of hydrophobic properties. In this connection simple ring substituted benzylamines could serve as model compounds. In this paper only benzylamine and its two derivatives 4-chloro and 4-isopropyl benzylamine are included†; binding parameters and partition coefficients are also listed in Table 2. Again as observed for phentermine/chlorphentermine an increase in hydrophobic forces is answered by an intensified interaction between the compounds and PCh. Further support for the significance of hydrophobic forces in the interaction is provided by the enhanced broadening of the proton signals in PCh/drug solutions after addition of NaCl (Fig. 4).

(2) *Relaxation rates of the proton signals of norfenfluramine as a function of its concentrations at constant PCh concentration.* In case of a fast exchange of the free and bound species, i.e. a reversible binding obeying the law of mass action, a decrease in line width broadening should occur if at constant PCh concentrations the drug concentration is increased. As an example the results with norfenfluramine are given in Fig. 5. It demonstrates that not the PCh or drug concentration but rather the ratio between both is important for the broadening of the signals. As postulated above an increase of drug concentration is answered by a decrease in line width broadening for the different spin systems.

(3) *Relaxation rates of the proton signals of the drugs studied in the presence of various lipids.* As additional lipids PCh_2, phosphatidylethanolamine (PE), digalactosyldiglyceride (DGD) and diacylglycerol have been studied. The binding affinities of chlorphentermine, fenfluramine and norfenfluramine obtained with the highly unsaturated PCh_2 were similar to those determined with PCh_1. As an example the results obtained with norfenfluramine and PCh_2 are given in the equations (8b) and (8c), which are almost identical with

* The statistics for eq. 1–8c are the standard errors of estimate, s, the correlation coefficient, r, and the F-test. The value in parentheses below the coefficients is the t-test.

† A large series of other benzylamine derivatives has been synthesized and the interaction with PCh was studied by Blaschke *et al.* [6].

Table 2. Proportionality factor, m, of the various spin systems (a, b, c, d) taken from the regression equations (1–7) and the partition coefficients for those compounds where a quantitative analysis of the interaction with PCh_1 could be performed (a = aromatic protons, b = methylen-protons, c = methyl protons, d = other systems, e = partition coefficients were calculated using the fragmental constants of Nys and Rekker [4]

Compound	m a	b	c	d	log $P_{octanol}$ e	Remarks (x)
Phentermine	x	x	x	—	2.45	slight interaction, no quantitative evaluation possible
Chlorphentermine	4.9	6.7	1.7	—	3.43	
Fenfluramine	1.6	3.2	x	2.2 (—CH_2—CH_3)	3.83	spin system too complex for analysis
Norfenfluramine	1.4	2.3	1.8	—	3.08	
Benzylamine	—	—	—	—	1.04	insignificant interaction
4-Chlorobenzylamine	x	1.3	—	—	2.02	spin system too complex for analysis
4-Isopropylbenzylamine	3.0	5.7	—	0.8 (—$CH(CH_3)_2$)	2.49	
Bromhexine. Metabolite I	x	4.8	—	x (—H)	2.31	spin system too complex for analysis
Chlorcyclizine	x	—	—	0.7 (—N—CH_3)	3.98	cyclizine itself was insoluble, spin system too complex for analysis

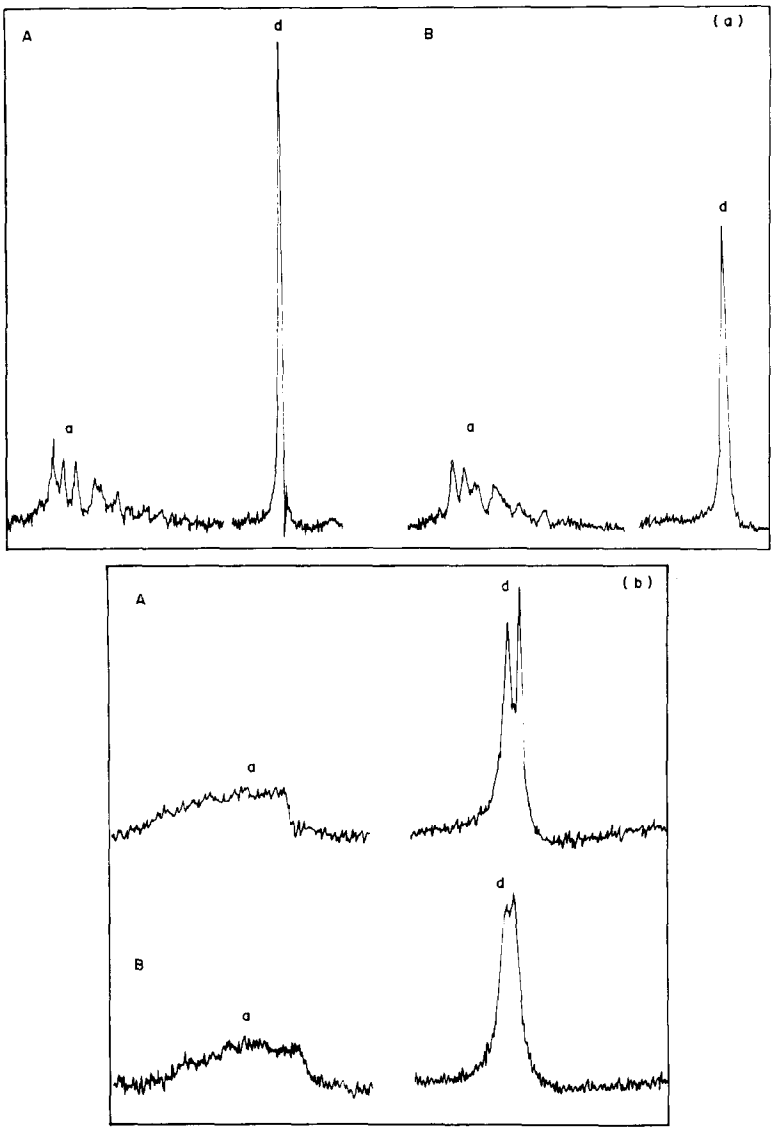


Fig. 3. (a) NMR proton resonance signals (a, = aromatic protons, 7,45 ppm, d = N—$(CH_3)_2$, 2,9 ppm) of amitriptyline (4×10^{-2} M) in the absence (A) and presence (B) of PCh_1 (2.4 mg/ml). (b) NMR proton resonance signals (a ~ 7,3 ppm, d = N—$(CH_3)_2$, 2.9 ppm) of 1-chloro-amitriptyline (4×10^{-2} M) in the absence (A) and presence (B) of PCh_1 (2.4 mg/ml).

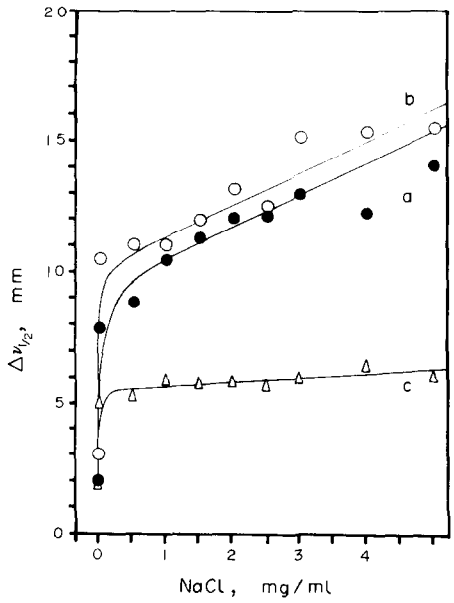


Fig. 4. Signal broadening ($\Delta\nu_{1/2}$, mm) of various spin systems of chlorphentermine (a, b, c) at constant PCh_1 concentration (1.2 mg/ml) as a function of increasing NaCl concentrations (0–6 mg/ml). The lowest symbols at zero NaCl concentration represent the control line width of the different signals in the absence both of PCh_1 and of NaCl.

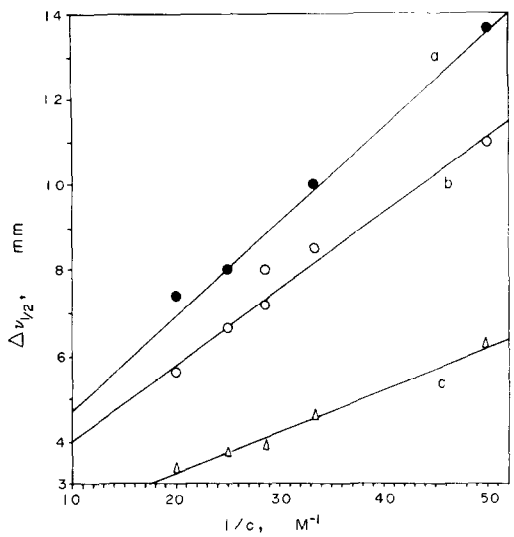


Fig. 5. Signal broadening ($\Delta\nu_{1/2}$, mm) of various spin systems of norfenfluramine (a, b, c) as a function of its inverse concentration at constant PCh_1 concentration (2.4 mg/ml).

equations (3b) and (3c) obtained with PCh_1.

	n	$y = (1/T_2)$ (mm) r	s	$x_2 = PCh_2$ (mg/ml) F	$S(\%)$	
Norfenfluramine:						
$y_b = 2.14\,x_2 + 4.06$ (20.5)	6	0.99	0.27	422	> 99.9	(8b)
$y_c = 1.3\,x_2 + 2.97$ (9.8)	6	0.98	0.35	96	> 99.9	(8c)

As for PE (in concentrations comparable to PCh) the addition of chlorphentermine caused the formation of insoluble complexes. This forced us to reduce the PE concentration to less than 0.1 mg/ml to keep the compounds in solution, however, under these limited experimental conditions no interaction between chlorphentermine and PE could be observed in NMR measurements. The other two lipids mentioned, which differ from PE and PCh by lack of a positive and negative charge could be dissolved in concentrations comparable to PCh. No interaction, however, with chlorphentermine occurred.

(4) *Influence of phentermine and chlorphentermine on the NMR spectrum of* PCh_2. In order to detect the

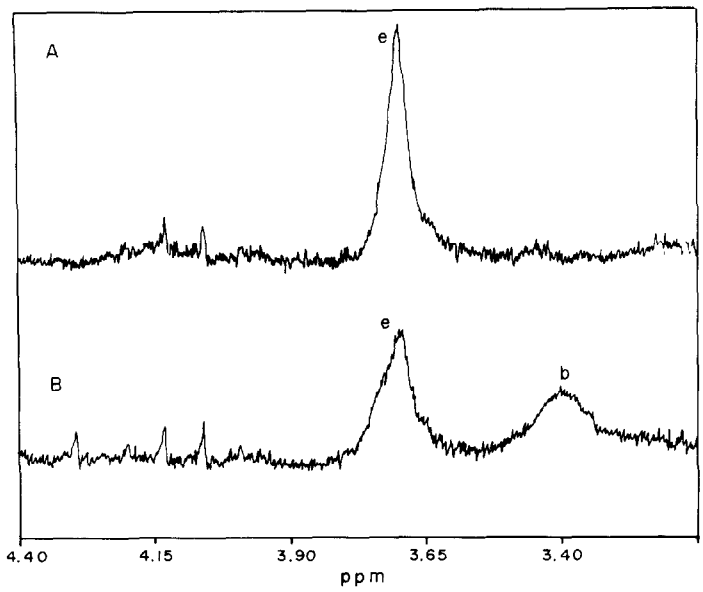


Fig. 6. NMR spectra of PCh_2 (24 mg/ml) in the absence (A) and presence (B) of chlorphentermine (13.2 mg/ml) e = $\overset{(+)}{N}(CH_3)_3$ proton signal of PCh_2 b = $-CH_2$ proton signal of chlorphentermine.

drug-induced alterations at the biological "binding site" (PCh_2), NMR spectra of sonicated PCh_2 solution (24 mg/ml) were recorded in the presence and absence of various phentermine and chlorphentermine concentrations (Fig. 6). The most obvious change for the PCh_2 spectra in the presence of the drug-induced alterations at the biological "binding ternary nitrogen (e). The large number of methylene groups ($(—CH_2)_n$) in the hydrocarbon chains gives rise to a broad complex signal. Because the methyl groups of the drugs show peaks in the same region a quantitative analysis of the drug interaction is not possible. This part of the spectra is therefore omitted in Fig. 6. The effects exerted by chlorphentermine on the NMR spectra of PCh are much more pronounced compared to phentermine. This can be taken as an additional proof for the specificity of the interaction reported.

(5) *Influence of cholesterol on the drug/PCh_2 interaction.* As an essential constituent of natural lipid mixtures cholesterol was included in this study in order to examine a possible additional role in drug/lipid interaction. The limited solubility of cholesterol in D_2O excluded a direct measurement of cholesterol/drug interaction phenomena.

The solubility of cholesterol can, however, be improved in the presence of PCh [7]. Therefore a graded amount of cholesterol was added to a PCh_2 solution (2.4 mg/ml) and sonicated. The results are summarized in Fig. 7. With increasing cholesterol concentrations the original drug/PCh_2 interaction is diminished. An interaction between cholesterol and PCh has already been described by Chapman and Penket [7]. The influence of cholesterol on the drug PCh interaction may therefore be of competitive nature (see Discussion).

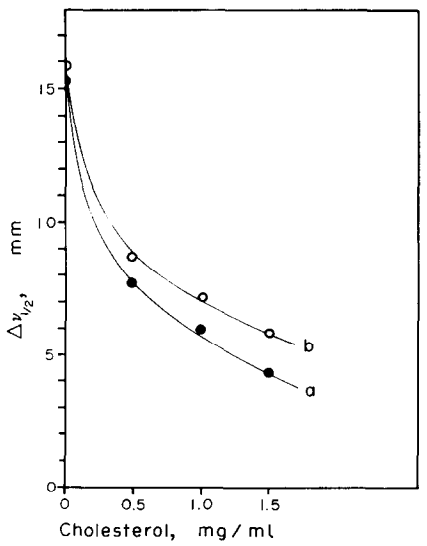


Fig. 7. Chlorphentermine/PCh_2 interaction (4×10^{-2} M/2.4 mg/ml) under the influence of cholesterol: The original line width broadening of the aromatic (a) and methylene (b) proton signals is decreased with increasing cholesterol concentration (0–1.5 mg/ml).

DISCUSSION

Chronic treatment with certain amphiphilic drugs as for instance chlorphentermine, chloroquine, 4,4-diethylaminoethoxyhexestrol (Table 1) causes an abnormal accumulation mainly of phospholipids in organs with a high content and/or rapid turnover of phospholipids in several species including man [1, 5]. The reason for this phenomenon might be either an interaction between the drugs and lipid metabolizing enzymes or between drugs and phospholipids as substrates. According to our previous investigations [2] the second possibility is more likely since no interaction between phospholipase A_2 and chlorphentermine could be detected in NMR binding studies. On the other hand a strong interaction between phospholipids and chlorphentermine has been observed. This is underlined by the fact that phosphatidylcholine and chlorphentermine precipitate under certain conditions. A remarkably strong precipitation occurred with another phospholipid, phosphatidylethanolamine, in the presence of even small concentrations of chlorphentermine. Similar observations have been reported by Hauser *et al.* [8] with local anaesthetics and phospholipids where the interaction of both components has been analyzed by NMR binding measurements.

Due to these observations one might speculate that the vesicular properties of the phospholipids could also be affected by the drugs in a different way (e.g. chlorphentermine—but not phentermine—causes a vesicular aggregation which gives rise to line width broadening). In this case, however, it should be expected that the different spin systems of the bound molecule show no differentiation in line width broadening.

If the mechanism of chlorphentermine-induced phospholipidosis originates in a chlorphentermine/phospholipid interaction similar binding phenomena in NMR should occur for the compounds discussed in this paper. In spite of the structural heterogeneity all compounds studied—with the exception of phentermine and benzylamine—exert a distinct but quantitatively different interaction with PCh. The highest affinity in this series of compounds was observed for chlorphentermine followed by 4-isopropylbenzylamine, fenfluramine and the other compounds (see Table 2).

The difference in binding affinity of chlorphentermine compared to fenfluramine and phentermine is paralleled by their different potencies in inducing phospholipidosis *in vivo* [9]. 4-Isopropylbenzylamine also impairs phospholipid metabolism, for example of isolated macrophages, in a typical way [10]. Besides this ranking in binding affinity for the several compounds, each drug molecule itself shows different binding patterns for its various molecular parts. Especially the aromatic ring protons and the adjacent methylene group show a larger increase in signal broadening compared to other spin systems. The essential contribution of the aromatic system and the adjacent aliphatic group count for the importance of hydrophobic binding forces. In the parent compounds, benzylamine and phentermine, these hydrophobic properties are too weak for a significant interaction with PCh and neither compound causes phospholipidosis *in vivo*. Introduction of proper substi-

tuents, however, reinforces hydrophobic binding of the resulting compounds and may give rise to the impairment of phospholipid metabolism. The role of hydrophobic interaction is supported by binding studies in the presence of increasing ionic strength (see Results, Fig. 4).

In general, one might also expect chemical shift changes for some nuclei of the drug molecule when it is bound to the "receptor". If the small molecule exchanges rapidly between the free and complexed environments, the observed spectral positions of the affected resonances will be shifted from their corresponding positions of the unassociated molecule by amounts, which depend on the chemical shifts of these nuclei in the complex as well as on the fraction of the drug molecules in the complex state. One might argue that the occurrence of a chemical shift indicates a more "stereospecific" interaction than the line-width changes observed in the experiments reported. Such chemical shift effect could result from electric fields due to polar groups, secondary magnetic field differences arising from induced magnetic moments in neighbouring atoms. In general, chemical shift changes are considerably smaller than the accompanying broadening of the resonance and are therefore more difficult to measure. In our experiments a chemical shift was not observed which may point to the fact that the specificity of the interaction is not pronounced and that hydrophobic interaction might be the dominant factor.

Another essential precondition for this type of interaction seems to be a protonated amino group in the aliphatic side chain. In this connection it might be of interest that analogue anionic compounds are not able to produce phospholipidosis.

The binding is determined also by the structural properties of the lipid: less polar lipids like DGD and diacylglycerol show no interaction with amphiphilic drugs. Differences in the saturation as in PCh_1 and PCh_2 do not significantly alter binding affinities. The interaction, however, with phosphatidylethanolamine seems to be intensified. Even at very low concentrations of both reactants a precipitation occurs thus preventing NMR measurements. The presence of charged groups in the lipid as binding partner might therefore be a prerequisite for interactions. Some support for an interaction at the polar region of the PCh is derived from NMR spectra of high PCh concentrations in the presence of chlorphentermine where the protons of the quaternary methyl groups of PCh are distinctly involved in binding (Fig. 6). The "antagonistic" action of cholesterol on the chlorphentermine/PCh interaction which occurs only if PCh is sonicated together with cholesterol may be explained by permeability changes of the lipid micelles (liposomes)/(an impermeability of phospholipid membranes due to the incorporation of cholesterol has been reported by Brockhoff [11]). If cholesterol is added after formation of the PCh/drug complex, no significant alteration of this complex can be achieved.

The results presented in this paper additionally support the postulated mechanism of drug-induced phospholipidosis. Depending on the structure, i.e. physicochemical properties of the drugs, these compounds do complex with the substrate (phospholipids) to a different degree thereby preventing the metabolic degradation of the lipids. Hydrophobic forces are essential for the complex formation provided that a cationic group is present in the side chain. A strong proportionality between the results of NMR binding measurements and the effects observed *in vivo* might not be expected for all amphiphilic drugs since drug metabolism and pharmacokinetics additionally have to be considered under *in vivo* conditions. Furthermore, different turnover rates, contents and pattern of phospholipids in different tissues and organs determine their affinity for these drugs and therefore the incidence of the side action reported.

REFERENCES

1. H. Lüllmann, R. Lüllmann-Rauch and O. Wassermann, *Dtsch. med. Wschr.* **98**, 1616 (1973).
2. J. K. Seydel and O. Wassermann, *Naunyn-Schmiedeberg's Arch. Pharmac.* **279**, 207 (1973).
3. H. Hauser and L. Iron, *Z. physiol. Chem.* **353**, 1579 (1972).
4. G. G. Nys and R. F. Rekker, *Chim. Thérap.* **8**, 521 (1973).
5. H. Lüllmann, R. Lüllmann-Rauch and O. Wassermann, *Critical Rev. Toxicol.* **4**, 185 (1975).
6. G. Blaschke, R. Jörgens and G. Claassen, *Deutsche Apotheker-Zeitung* **114**, 1675 (1974).
7. D. Chapman and S. A. Penkett, *Nature* **211**, 1304 (1966).
8. H. Hauser, S. A. Penkett and D. Chapman, *Biochim. biophys. Acta* **183**, 466 (1969).
9. R. Lüllmann-Rauch and G. H. Reil, *Naunyn-Schmiedeberg's Arch. Pharmac.* **285**, 175 (1974).
10. D. Drenckhahn and L. Kleine (personal communication).
11. H. Brockerhoff, *Lipids* **9**, 645 (1974).
12. A. R. Johnson and R. B. Stocks in *Biochemistry and Methodology of Lipids* (Eds. A. R. Johnson and J. B. Davenport) p. 195. Wiley, New York (1971).