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THE BINDING OF DRUGS TO DIFFERENT POLAR LIPIDS *IN VITRO*

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Abstract—For evaluation of the physico-chemical basis underlying the drug-induced generalized lipid storage disease, the equilibrium distribution of radioactively labelled amphiphilic drugs between a water phase and liposomes was determined. Liposomes were prepared from sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS); the extent of binding of the drugs occurred in increasing order SM~PC<PE<PS. A strong correlation was found between the octanol–water partition coefficient and the PC–water coefficient for monovalent cationic drugs; the absolute values of both partition coefficients resembled each other very closely, suggesting that the hydrophobic forces are mainly responsible for the binding of amphiphilic drugs to PC. The higher extent of binding to PS does not result from higher affinities of the drugs to PS but rather from the higher capacity of PS-liposomes as compared with that of PC- or SM-liposomes. The divalent cationic drug chloroquine displayed particularly high binding to the anionic lipids PS and to gangliosides as compared with the monovalent drugs. This observation might help to explain the ultrastructural and biochemical findings that chloroquine induces a remarkable storage of anionic lipids upon chronic treatment of animals.

In recent years a side effect has been described of a number of cationic amphiphilic drugs which induce lysosomal storage of polar lipids (for review see [1, 2]). In ultrastructural studies accumulation of lamellated or crystalloid cytoplasmic inclusion bodies containing polar lipids was observed in various cell types. A direct interaction between the amphiphilic drugs and the polar lipids is considered to be responsible for the impaired degradation and for the accumulation of the "lipid–drug-complexes" within lysosomes. This concept is based upon NMR-studies [3], upon electrophoretic and enzymatic studies [4], and upon biochemical determinations [5]. The development of the drug-induced lipidosis *in vivo* does not permit quantitative conclusions as to the molecular interactions between the drugs and the polar lipids. It is, therefore, the aim of the present study to investigate, under well-defined *in vitro* conditions, the interaction of a number of cationic amphiphilic drugs with different polar lipids, in order to obtain information of the affinity and binding capacity. Four drugs (chloroquine, phentermine, chlorphentermine, 1-chloro-amitriptyline) which have previously been shown to induce lysosomal storage of polar lipids were investigated. In order to cover a wide range of hydrophobicity, several other cationic amphiphilic drugs were included. For sake of comparison, some anionic and neutral drugs were also used in the study. Particular interest was focused upon the divalent cationic drug chloroquine, since within the present context, it differs somewhat from the monovalent cationic compounds with respect to the ultrastructure of the cytoplasmic inclusions and to the distributional pattern of the lipidosis induced [6, 7].

METHODS

1. *Compounds*

Polar lipids. Phosphatidylcholine was prepared chromatographically from crude egg yolk lecithin by the method of Marsh and Holzbach [8]. Phosphatidyl ethanolamine was obtained from Applied Science Laboratories (No. 22022, bovine brain), phosphatidylserine, sphingomyelin and ganglioside from Sigma Chemie GmbH Munich (No. P 6641, No. S 7004 and No. G 2375, bovine brain).

The phospholipids yielded single spots in thin-layer chromatography using a solvent consisting of $CHCl_3$, CH_3OH and H_2O (65:35:4). Under the same conditions the batch of gangliosides was separated into at least four different fractions, as expected from the methodical procedure for their isolation [9]. The batch was used without further purification.

Drugs. The drugs studied in the present work are listed in Table 1 in the order of their lipophilicity; some physico-chemical parameters are also given. The drugs contained in Table 1, were labelled with tritium or ^{14}C from Amersham Buchler, Braunschweig. The labelled drugs were checked for impurities by radiochromatography (Packard Scanner, Modell 7201) and were chromatographically purified if contamination amounted to more than 10 per cent.

Buffer. The buffer used in all experiments contained 100 mM NaCl, 2 mM l-histidine (Sigma No. H 8000), 2 mM TES [Tris-(hydroxymethyl)-methyl-2-aminoethansulfonic acid] (Sigma No. T 1375), and 0.1 mM EDTA (Merck, Darmstadt), and was adjusted to pH 7.4 [10]. Its capacity is sufficient over a range from pH 5 to pH 10. The NaCl concentration of 100 mM in the buffer was chosen to avoid purely

Table 1.

Drug	Source	pK_a	log P (Octanol/H_2O)	log P' (Octanol/H_2O)
1. Atenolol	ICI-Pharma, Plankstadt	9.6	0.17	−1.94
2. Phenobarbital	Bayer AG, Leverkusen	7.5	1.43	1.13
3. Atropine	Merck, Darmstadt	9.6	1.76	−0.44
4. Dexametasone	Merck, Darmstadt		1.76	1.74
5. Phentermine	Mack, Illertissen	10.1	1.90	−0.8
6. Metroprolol	Astra Chemicals GmbH, Wedel/Holst.	9.6	2.34	0.04
7. Carticaine	Hoechst AG, Franfurt	8.3	2.41	1.50
8. Verapamil	Knoll AG, Ludwigshafen	8.4	2.51	1.70
9. Chlorphentermine	Troponwerke GmbH, Köln	9.6	2.60	0.4
10. Phenylbutazone	Ciba Geigy GmbH, Wehr (Baden)	4.4	3.04	1.41
11. Propranolol	ICI-Pharma, Plankstadt	9.6	3.14	1.41
12. Dexetimide	Janssen GmbH, Düsseldorf	8.7	3.55	2.36
13. Phenprocoumon	Hoffmann-La Roche AG, Grenzach-Wyhlen	5.0	3.62	1.23
14. Chloroquine	Bayer AG, Leverkusen	10.1; 8.1	4.63	1.23
15. 1-Chloro-amitriptylin*	Hoffmann-La Roche AG, Grenzach-Wyhlen	9.4	5.55	3.55

Compilation of labelled drugs used in the present study listed according to increasing lipophilicity (log P). Values for pK_a, log P and log P' are kindly supplied by Dr.P.B.M.W.M. Timmermans, Department of Pharmacy, Division of Pharmacotherapy, University of Amsterdam, Amsterdam, Netherlands.

* Experimental compound.

electrostatic influences upon the lipid array by the cationic drugs used in the concentration range from 0.001 to 3 mM.

2. *Preparation of liposomes*

The polar lipids, dissolved in chloroform, were dried under vacuum for at least 12 hr, until the tube had attained constant weight. In order to obtain liposomes, 3–6 ml of the buffer, saturated with nitrogen, were added under permanent N_2 flow, and the tubes were shaken by hand for 2 min. The coarse suspension thus formed was sonicated at 37° for 2 min at 75 W (Labsonic 1510, 0.5 cm microtip). Care was taken not to surpass a temperature of 39°.

The final lipid concentrations were adjusted to 0.25 mM (phosphatidylserine), 0.5 mM (phosphatidylethanolamine) and 1.0 mM (phosphatidylcholine, sphingomyelin, gangliosides), respectively.

3. *Determination of the partition coefficients*

Lipid suspensions were mixed with equal volumes of a drug solution in buffer. The samples were kept under nitrogen at 37° for 14 hr to warrant an equilibrium distribution. In case of sphingomyelin, additional control experiments were performed at 50° to ascertain bilayer formation. The relative binding of the drugs investigated were found to be in the same order as that obtained at 37°, although the absolute values increased by 50 per cent at the higher temperature. In order to separate the phases, the samples were centrifuged at 37° for 30 min at an average acceleration of 135,000 g ("Airfuge®" Beckman). Samples containing no liposomes but only the drug were subjected to the same procedure. Radioactivity in the supernatants was determined by liquid scintillation counting (Packard Tri Carb Counter 3380). Consequently the apparent partition coefficients P' could be calculated according to Bowley *et al.* [11] by the following equation:

$$\frac{c\text{-}c_f}{c_f} \cdot \frac{\text{weight of buffer}}{\text{weight of polar lipid}} = P'$$

(c: concentration of the drug in the supernatant of liposome-free samples, c_f: concentration of the drug in the supernatant of liposome containing samples).

For sake of comparison some equilibrium dialysis experiments were performed. A Teflon cell dialysator was charged with the suspensions described before. The results obtained with this method did not differ from those of the centrifugation procedure and are therefore not further mentioned.

4. *Phosphate determination*

Since the sedimentation of polar lipids is not complete after centrifugation, the amount of lipids remaining in the supernatant has to be taken into account. Consequently the phospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and sphingomyelin) in the supernatant had to be determined. After acid hydrolysis, inorganic phosphate was measured by the method of Chen *et al.* [12]. The gangliosides were determined semi-quantitatively by thin-layer chromatography. The area and intensity of the spots obtained from the suspension and from the supernatant were compared. The amount of gangliosides which could not be sedimented, proved to be less than 10 per cent of the initial value.

RESULTS

1. *Control experiments*

The interaction between liposomes and drugs will result in a decrease of the drug concentration in the

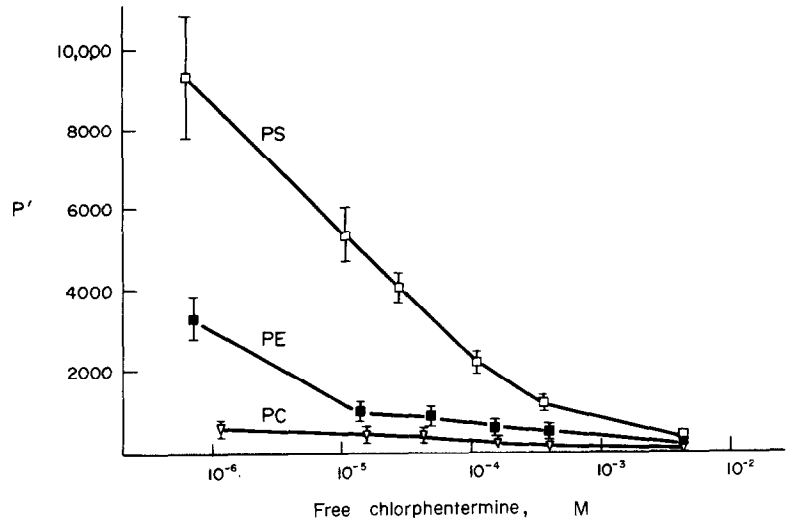

Fig. 1. The partition coefficient P' (ordinate) of chlorphentermine for phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) dependent upon the concentration of the free chlorphentermine (abscissa). The symbols represent means ± S.E.M. n for PE and PC = 6, n for PS = 14.

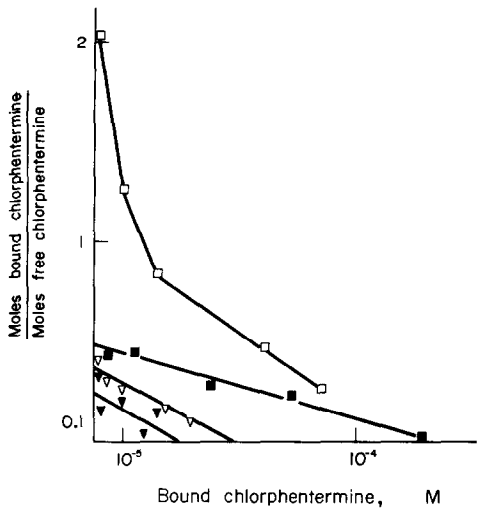

Fig. 2. The binding of chlorphentermine to phosphatidylserine (□), phosphatidylethanolamine (■), phosphatidylcholine (▽) and sphingomyelin (▼). The fraction bound to free chlorphentermine (ordinate) is plotted vs the concentration of bound chlorphentermine (Scatchard-plot).

supernatant. To enable a correct determination of the change, the concentration of the labelled drugs in the supernatant should be altered by the lipids by at least 10 per cent and by not more than 90 per cent. At drug concentrations between 10^{-5} and 10^{-4} M, the following lipid concentrations met the requirement: 10^{-3} M phosphatidylcholine (PC), 10^{-3} M sphingomyelin (SM), 0.25×10^{-3} M phosphatidylserine (PS), 0.25×10^{-3} M phosphatidylethanolamine (PE). These lipid concentrations were kept constant throughout all experiments.

Since polar lipids cannot be sedimented quantitatively, we checked whether or not the drug concentration influenced the non-sedimentable lipid fraction. Within a concentration range of 10^{-5} M and 5×10^{-3} M chlorphentermine, the non-sedimented fraction of PC remained constant at about 20 per cent.

2. *Binding curves*

In a concentration range between 10^{-6} and 3×10^{-3} M, chlorphentermine was bound to PS to a much higher degree than to PE or PC. A presentation of the experimental data expressed as P' (apparent partition coefficient) yielded curves which

Table 2.

Polar lipid	Drug	h	r
Phosphatidylserine	chlorphentermine	0.78	0.99
Phosphatidylethanolamine	chlorphentermine	1.08	0.99
Phosphatidylcholine	chlorphentermine	0.94	0.99
Sphingomyelin	chlorphentermine	0.96	0.93
Phosphatidylserine	phentermine	0.95	0.99
Phosphatidylethanolamine	phentermine	0.90	0.93

Hill coefficients (h) of the binding curves and their linear correlation coefficients (r) as obtained from Hill plots.

Table 3.

Polar lipid	Drug	$k[10^{-4}M]$	n_{max} moles drug / moles lipid	r
Phosphatidylserine	chlorphentermine	2.17	0.67	0.99
Phosphatidylethanolamine	chlorphentermine	1.02	0.27	0.98
Phosphatidylcholine	chlorphentermine	1.26	0.05	0.95
Sphingomyelin	chlorphentermine	1.28	0.03	0.71
Phosphatidylserine	phentermine	1.49	0.19	0.98
Phosphatidylethanolamine	phentermine	1.46	0.03	0.93

Half-saturation concentration (k), maximum binding capacity (n_{max}) and the correlation coefficients (r) of the binding curve in a Scatchard plot.

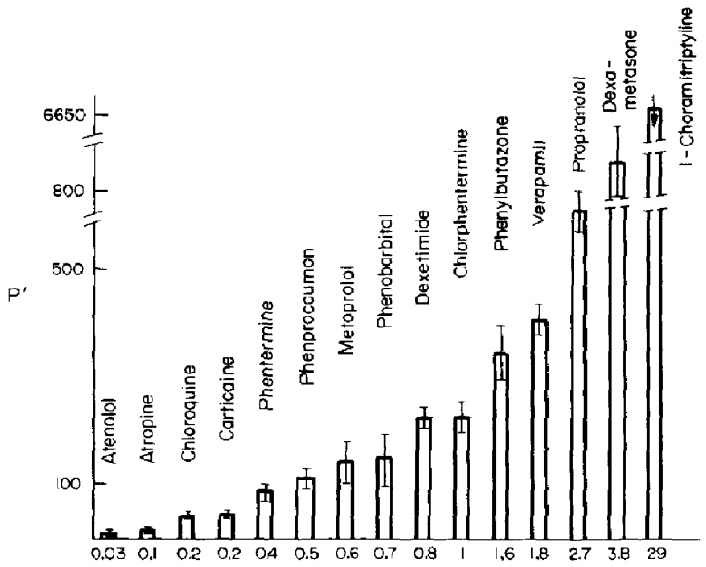

Fig. 3. The partition coefficients P' of 15 drugs for phosphatidylcholine at a drug concentration of 2×10^{-4}M. The numbers under the columns demonstrate the relative partition coefficients of the different drugs in comparison to that of chlorphentermines equal 1.0 (means ± S.E.M.; $n = 6$).

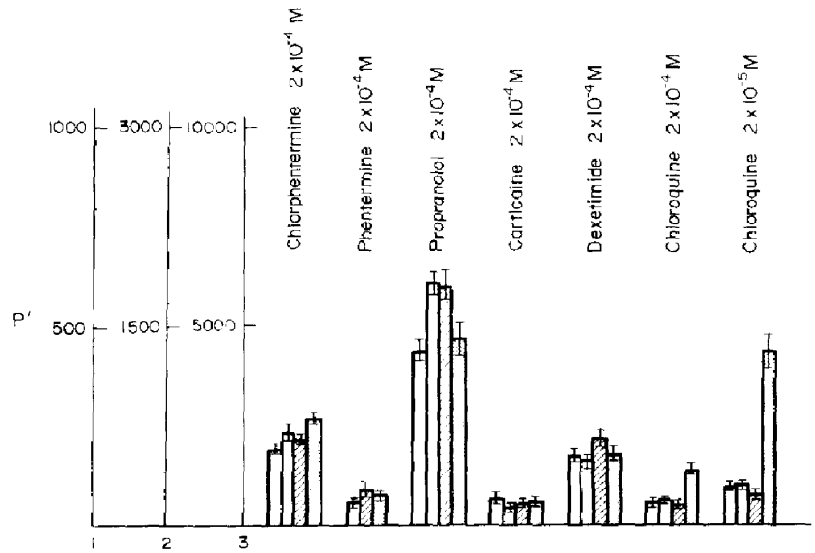

Fig. 4. The partition coefficients of six drugs for different phospholipids. For sake of comparison different ordinate measures are chosen (mean ± S.E.M.). 1 stands for open columns (sphingomyelin left, phosphatidylcholine right). 2 stands for hatched columns (phosphatidylethanolamine). 3 stands for stippled columns (phosphatidylserine). The P' value of phentermine for sphingomyelin is omitted, since it could not be determined due to the minute binding.

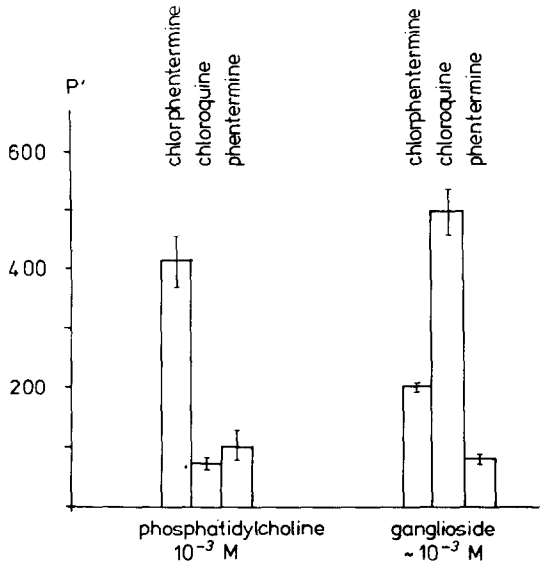

Fig. 5. Comparison between the partition coefficients P' of chlorphentermine, chloroquine and phentermine for the neutral phosphatidylcholine and for the anionic gangliosides at drug concentration of 2×10^{-5} M (mean ± S.E.M.).

indicate a saturation process (Fig. 1). Similar curves were obtained for phentermine and chlorphentermine with the other phospholipids. The Scatchard plot (Fig. 2) shows that only one of the binding curves failed to give a straight line as expected from simple adsorption processes. The binding of chlorphentermine to PS resulted in a hyperbola suggesting a co-operative interaction. In order to quantify the cooperativity the Hill coefficients were evaluated using a Hill plot. The resulting Hill coefficients for chlorphentermine and phentermine, and for different polar lipids are listed in Table 2. All Hill coefficients were found to be close to 1 except the coefficient for the synergism between chlorphentermine and PS, which was estimated to amount to 0.78, indicating negative synergism. Taking the Hill coefficient into account, the half-saturation concentrations k and the maximum binding n_{max} were evaluated (Table 3). The k values differed only slightly (range $1.02–2.17 \times 10^{-4}$ M), whereas the n_{max}-values varied from 0.03 to 0.67 moles of drug per mole of polar lipid.

3. *Affinity of several drugs to phosphatidylcholine (PC)*

In addition to chlorphentermine and phentermine, thirteen other compounds were studied with respect to their binding to PC. They were selected to cover a wide range of lipophilia; they belong to different groups of drugs. The binding was determined at a drug concentration of 2×10^{-4} M. The apparent partition coefficients P' are listed in the order of increasing tendency to bind to PC (Fig. 3). The relative affinities of the compounds were compared with that of chlorphentermine (set equal 1). The relative values ranged from 0.03 for atenolol to 29 for 1-chloroamitriptyline thus covering an increase of about 1000 times.

4. *Affinity of some compounds to four different polar lipids*

The bindings of chlorphentermine, phentermine, propranolol, carticaine, dexetimide and chloroquine to PC were compared with the binding towards other polar lipids. All drugs except chloroquine became bound to PC, SM, PE and PS in the relative order of approximately 1:1:3:10. Chloroquine, however, displayed a much higher binding to PS, i.e. 30–60 times higher than to PC. This relationship is graphically demonstrated in Fig. 4.

The unexpected high binding of chloroquine to PS might result from the negative charge which distinguishes PS from the zwitterionic lipids PC, PE and SM. Therefore, we extended the study to another class of negatively charged polar lipids, i.e. to gangliosides. A comparison was made between the binding of chlorphentermine, phentermine and chloroquine, towards gangliosides and towards PC (Fig. 5). The binding of chloroquine to gangliosides exceeded that of phentermine by six fold, whereas the binding of the two compounds to zwitterionic polar lipids was similar.

5. *Correlation between lipophilicity and binding to phosphatidylcholine (PC)*

An attempt was made to correlate the PC-binding with the water/octanol partition of the uncharged drug molecules [Fig. 6(a)]. A strong correlation between the polar lipid binding [log P' (PC)] and the partition coefficient (log P octanol/H_2O) was found only for the monovalent cationic drugs. The regression line displays a slope of 0.59, a coefficient $r = 0.95$, and an intercept at log $P' = 0.62$. When the PC binding was correlated with the apparent partition coefficient (log P' octanol/H_2O), a moderate correlation was obtained even if all investigated compounds were included [Fig. 6 (b)]. The regression line has a slope of 0.42 and a coefficient $r = 0.80$. This correlation could not be improved by taking only the monovalent cationic drugs into account. The evaluation suggests that hydrophobicity is the main determinant for the binding to PC. The interpretation is supported by results obtained for local anesthetics [13, 14], for a series of substituted benzylamines [15], and for some pairs of drugs differing only in the hydrophobic moeity [3].

DISCUSSION

Out of the 15 compounds investigated, two drugs, i.e. phentermine and chlorphentermine were studied over the entire concentration range which permitted reliable measurement. The two drugs differ only by the para-chloro-substitution resulting in an increase of the octanol/H_2O partition coefficient from 1.9 to 2.6 (log P). Comparison of the bindings of phentermine and of chlorphentermine towards several polar lipids revealed that the difference in binding were mainly due to differences in the maximum capacity rather than to different affinities expressed as half saturation concentrations (see Table 3).

Out of the 15 compounds, six drugs were studied with respect to the binding to different polar lipids. It could be shown that the lipids bound in the following order: PC = SM < PE < PS (about 1:3:10).

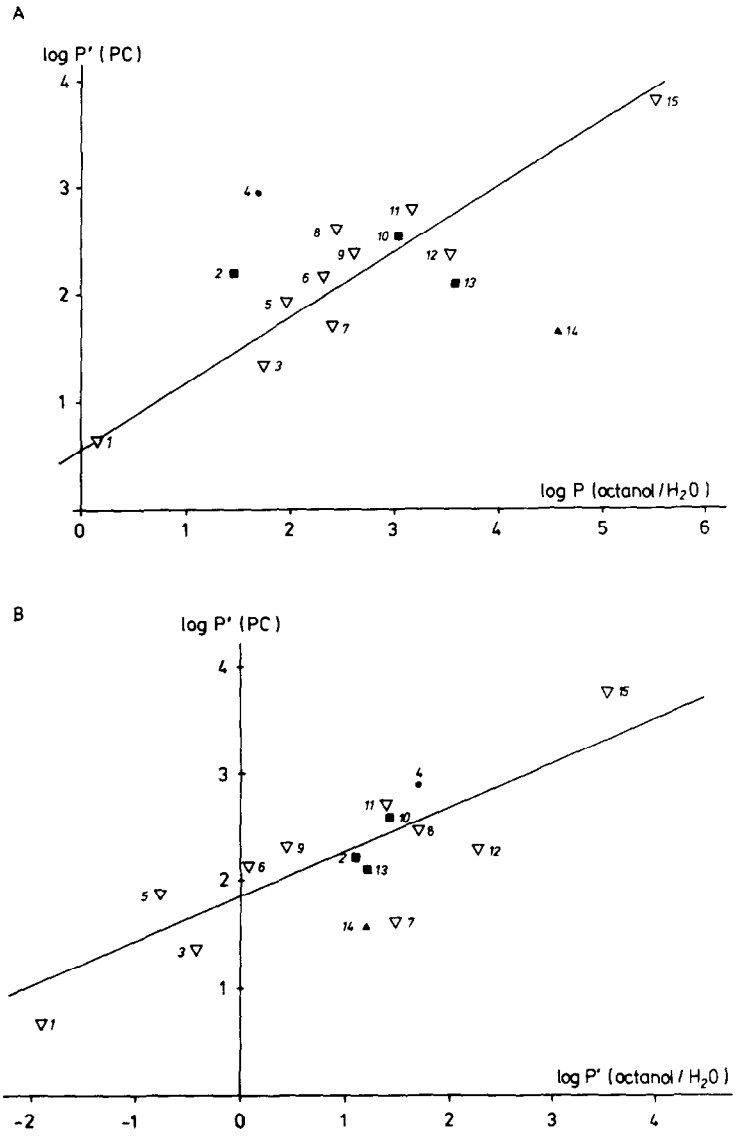

Fig. 6. (a). Correlations between the partition of 15 drugs into phosphatidylcholine, log P' (PC), and the partition of the uncharged compound into octanol, log P (octanol/H_2O). (b). Correlation between the partition of 15 drugs into phosphatidylcholine, log P' (PC), and the partition of the drug at pH 7.4 into octanol, log P' (octanol/H_2O). Drugs are numbered according to Table 1. ▽ : monovalent cation. ▲ : divalent cation. ■ : monovalent anion. ● : neutral compound. The straight lines in A and B represent the calculated linear regressions for the monovalent cations (▽).

The divalent cationic drug chloroquine, possessing pK_a-values of 8.1 and 10.1 [16], displayed a much higher affinity to the anionic polar lipids PS and gangliosides.

All compounds were investigated with respect to their binding to PC. For the monovalent cationic drugs, a strong correlation was found between the lipid binding and the octanol/H_2O partition coefficient log P [(Fig. 6 (a)]. Using the apparent coefficient log P', instead of log P, a moderately fitting regression could be calculated including all compounds irrespective of their charge [Fig 6(b)]. It should be mentioned that the divalent compound chloroquine became bound less than expected from its log P, whereas the uncharged compound dexamethasone became attached to PC to a higher degree than charged drugs with similar log P.

In the following we attempt to interpret the interaction between the drugs and the polar lipids. Both the polar lipids and the investigated drugs possess amphiphilic properties, thus two kinds of interaction are conceivable: electrostatic and/or hydrophobic forces could be responsible for complex formation. One has to distinguish between the binding of drugs to zwitterionic polar lipids (PC, PE, SM) not possessing a net charge, and to polar lipids with a negative net charge (PS, gangliosides). In the former case the hydrophobicity of the monovalent cationic drugs will almost entirely determine the degree of binding as demonstrated for PC. It may be noted

that the log P (octanol/H_2O) and the log P' (PC) closely resemble each other suggesting the prevalence of identical determinants governing the two distribution processes. Thus the charge of the drugs does not essentially add to the binding towards PC.

Concerning the binding of monovalent cationic to negatively charged polar lipids, a remarkably high complexation was found in comparison with neutral polar lipids. One is tempted to consider ionic bonds to be responsible for the higher binding. This should lead to a higher stability of the complexes resulting in a lower half-saturation concentration; however, this was not observed (see Table 3). However, in contrast, the interactions between anionic polar lipids and cationic drugs had half saturation concentrations similar to those found with neutral lipids, whereas the binding maxima were higher. The essential difference between the neutral PC and PE, and the anionic PS concerns the hydrophilic head group, whereas the apolar moieties are basically identical. The polar head group containing the serine moiety possesses a larger hydration radius than those of the neutral polar lipids; the occupied areas are further enlarged by electrostatic repulsion between adjacent serine head groups [17]. This implies that the conformational arrays of the polar lipid molecules differ with respect to the intercalated spaces. The larger the volume not directly occupied by the polar lipid molecules, the larger the volume which is available for the intercalation of the cationic drugs. Thus PC and PS will considerably differ in their capacities (n_{max}) to bind amphiphilic compounds. This view is supported by the present finding of negative synergism exhibited by the binding of chlorphentermine towards PS. The positive charge of the drug located in the region of the negative head groups will diminish the repulsion between the polar moieties of the lipid molecules and thus reduce the space for intercalation. Such an effect has already been demonstrated for inorganic cations [17].

In conclusion the interaction between a number of monovalent cationic amphiphilic drugs and several polar lipids can be considered as partition of the drugs between a water phase and a dispersed lipid phase. In case of monovalent compounds the charges seem to play only a minor role. The divalent cationic drug chloroquine possesses an unexpectedly high affinity to negatively charged polar lipids which might suggest that the additional charge promotes the binding.

The interaction of cationic amphiphilic drugs and polar lipids presently demonstrated *in vitro* is thought to be the basic mechanism underlying the drug-induced lipid storage disease observed in animals and humans (for review see [1, 2]). On the other hand, the present data do not permit direct conclusions as to the lipidosis-inducing potencies of the drugs under biological conditions. In intact organisms drug metabolism and drug distribution may obscure the potency to induce lipidosis. Furthermore, it is not possible to give sufficiently high doses of drugs with powerful main pharmacological actions to induce lipidosis. The exceptionally high affinity of chloroquine to PS and gangliosides might help to explain why this drug induces a storage disease of slightly different ultrastructural appearance and of different distributional pattern, i.e. particularly affecting ganglionic cells [6, 7, 18, 19]. A particular tendency of chloroquine to induce accumulation of gangliosides [18, 19] and of the anionic lipids bis-(monoacylglycero)-phosphate and phosphatidylinositol [20, 21] has also been demonstrated.

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