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THE DEPENDENCE OF THE LIPID BILAYER MEMBRANE: BUFFER PARTITION COEFFICIENT OF PENTOBARBITONE ON pH AND LIPID COMPOSITION

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1 The membrane/buffer partition coefficient of [^{14}C]-pentobarbitone has been determined as a function of the lipid composition of bilayer membranes.

2 A new technique based on ultrafiltration gave comparable results to conventional techniques but required less time for equilibration.

3 The membrane/buffer coefficient was independent of pentobarbitone concentration in the range studied.

4 The apparent partition coefficient varied with pH and was a linear function of the degree of dissociation of pentobarbitone.

5 Both the charged and uncharged forms of pentobarbitone partitioned into the membrane, the latter to a much greater extent than the former.

6 At low pH the highest partition coefficient observed was in egg phosphatidylcholine bilayer membranes.

7 Incorporation of cholesterol or phosphatidic acid into phosphatidylcholine membranes greatly reduced the partition coefficient.

8 High pressures do not greatly change these partition coefficients.

Introduction

The lipid solubility of barbiturates is one of the important controlling factors of their pharmacodynamics (Brodie, Kurz & Schanker, 1960) and may also account for their central depressant properties (Seeman, 1972). In spite of this, there is no systematic information on the partitioning of barbiturates into membranes of defined composition, and most studies requiring such information have relied upon partition coefficients from simple solvents. The reliability of the latter manoeuvre is questionable, however, for depending on the choice of solvent, the absolute value of the partition coefficient may vary by orders of magnitude, and even the ranking order of a number of barbiturates in different solvents may change (Kakemi, Arita, Hori & Konishi, 1967). In the face of these uncertainties a systematic study of the dependence of the partition coefficients of barbiturates on the lipid composition of membranes seems long overdue and ought to provide a firmer foundation for the assessment of both their pharmacodynamics and the specificity of their action on cellular structures.

In this paper we report the membrane-buffer partition coefficients of 5-ethyl-5-(1-methylbutyl) barbituric acid (pentobarbitone) in lipid bilayer membranes of various compositions. Lipid bilayer membranes were chosen, rather than a series of biomembranes, both because their lipid composition can be systematically varied and because they avoid the problem of simultaneous lipid and protein interactions with the drug. Since barbiturates are weak acids with pKs in the physiological range, their pharmacodynamics (Brodie *et al.*, 1960) and anaesthetic action (Narahashi, Frazier, Deguichi, Cleaves & Ernam, 1971) are pH-dependent, and we have therefore also examined the influence of pH on the membrane-buffer partition coefficients.

Methods

Two forms of lipid bilayers could be used for these studies. First, strong agitation or brief sonication of an aqueous lipid dispersion results in the formation of a large number of concentric spherical lipid bilayer shells separated by aqueous compartments. Second, upon more prolonged sonication these multi-lamellar vesicles form monolamellar vesicles with single internal aqueous compartments (Bangham, 1972). Both types of vesicles have been used in solubility studies. The monolamellar vesicles have the advantages of being of well defined size and structure and of requiring that the agent in question only penetrate one phospholipid bilayer to achieve complete equilibration with the lipid and internal compartment. On the other hand, these vesicles are not readily sedimented in the centrifuge and consequently a dialysis bag has been employed in the past in order to determine the buffer concentration of the agent in equilibrium with the membrane suspension. This increases the equilibration time and reduces the sensitivity of the method, and in consequence use of monolamellar vesicles in partitioning studies has been restricted to agents of high partition coefficients such as steroids (Heap, Symons & Watkins, 1970). The readily centrifuged multilamellar vesicles have thus been used in most studies previously reported. However, we found them to impose unacceptably long equilibration times, particularly in view of the possibility of lipid oxidation. To overcome these shortcomings we describe the use of an ultrafiltration method which allows partition coefficients to be studied in monolamellar vesicles with a sensitivity comparable to that generally obtained with multilamellar vesicles.

Materials

Phospholipids from egg yolk were obtained from Lipid Products, U.K., and were tested for purity by thin layer chromatography. Cholesterol was Sigma's chromatographic grade and was recrystallized in methanol twice before use to yield a m.p. of 148–9°C *in vacuo*. Radioisotopes were from New England Nuclear.

Procedure

About 5 μmol of the appropriate lipids for each ml of final suspension were mixed in organic solvents and evaporated to dryness. They were resuspended in 0.15 M KCl appropriately buffered (e.g. 10 mM Tris-HCl) and sonicated to clearness in a graduated centrifuge tube under nitrogen at 20°C in a sonicating bath (Heat systems, Model 5 × 5, 40 kHz). Aliquots of this suspension were added to buffer solution containing 0.2–0.8 mM pentobarbitone including 0.1 μCi of [^{14}C]-pentobarbitone. The pH was checked before and after incubation; adjustment of the pH was particularly necessary with suspensions containing phosphatidic acid. Equilibration, which was actually achieved within an hour or so, was normally allowed to proceed overnight in magnetically stirred bottles which were sealed under nitrogen and placed in a water bath maintained at 25.0 ± 0.05°C. The oxidation index (Klein, 1970) of the phospholipids did not increase during this procedure. An aliquot of the equilibrated suspension was taken for phosphate determination (McClare, 1971). A second aliquot was filtered through an ultrafilter (Amicon, XM-50) in a 10 ml filtration cell (Amicon model 12) thermostated in the water bath, the first 0.5 ml of filtrate being discarded and the remainder analysed by liquid scintillation counting in a Nuclear Chicago, Mark II, system. Control solutions, not equilibrated with lipid vesicles, were treated in the same way to yield the initial or total pentobarbitone concentration. Corrections for adsorption on the filter were less than 2% of the final partition coefficient. Two controls were performed in early experiments to ensure that the filter was achieving complete separation. First, no phosphate could be detected in the filtrate and second, when [^{14}C]-cholesterol was incorporated in the vesicles in the absence of [^{14}C]-pentobarbitone, no radioactivity above background was detected in the filtrate.

A few experiments were carried out at elevated pressure. Equilibration was carried out in small stainless steel bombs. Since filtration at high pressure was not possible, 0.5 ml of liposome suspensions in dialysis bags were equilibrated in 10–15 ml of buffer containing pentobarbitone in glass tubes sealed with a serum cap and stirred magnetically. One atmosphere controls were treated similarly in an unpressurized bomb. Hydrostatic pressure was raised by hand pump and measured on a bourdon tube gauge to ± 5%. Equilibration was for 18 hours. Depressurization, opening the bombs and removal of the dialysis bags was achieved always within 1 to 5 minutes. The rate of equilibration of pentobarbitone through the dialysis bag was too slow to introduce errors during this manoeuvre.

The partition coefficient was calculated from the difference between the initial and final buffer concentration and the concentration of lipid present in the suspension. The coefficient is expressed as the ratio (mol of pentobarbitone per g of lipid/mol of pentobarbitone per ml of buffer). The molecular weight of egg phosphatidylcholine was taken as 780 daltons. However, in Figures 1 and 2 only the partition coefficient is given per g of phospholipid (omitting any incorporated cholesterol), this being the direct experimental variable.

Results

A comparison between the partition coefficient obtained with the ultrafilter and that obtained with the conventional centrifugation method was performed using multilamellar vesicles of phosphatidylcholine at pH 7.9 in both cases; these methods yielded values of 98 and 100, respectively, which are not detectably different. Comparison of single shelled phosphatidylcholine vesicles at pH 7.9, separated from the buffer either by filtration or by a dialysis bag, yielded values of 104 and 105 respectively.

The measured membrane/buffer partition coefficient is almost independent of the pentobarbitone concentration in the buffer over the range 0.15–2.0 mM (Figure 1). The small, but consistent, decrease observed is statistically significant ($P < 0.002$) only when the data are pooled. The data show that the deviations from the ideal are very slight in this concentration range where the mol fractions of pentobarbitone dissolved in the lipid range from about 0.2 for phosphatidylcholine to 0.02 for phosphatidylcholine : cholesterol 1:1.

The partition coefficient (λ) of the acid and its anion may be derived by measuring the apparent λ over a range of pH. In Figure 2 the results of such studies are presented for two membranes. The relation between the apparent λ and the degree of dissociation is linear within experimental error, suggesting that the acid and its anion interact independently with the membrane. The partition coefficient for the acid, λ^{HA}, and the anion λ^{A-}, derived from the intercepts of such plots are given in subsequent figures. The magnitude of the partition coefficient for the anion was not entirely expected and was accordingly checked in experiments with multilamellar vesicles, which were separated from buffer after equilibration by either centrifugation or ultrafiltration. In addition to scintillation counting a spectroscopic assay of the barbiturate was also employed (Williams & Zak, 1959). These experiments gave identical results with our usual method, yielding for phosphatidylcholine vesicles a λ^{A-} of 9 ± 2.5. Our value of λ^{A-} is thus not an artifact of the filtration procedure, nor attributable to possible chemical degradation products of the labelled barbiturate.

The effect of varying the lipid composition of the bilayer membrane was next examined. Cholesterol was incorporated into phosphatidylcholine bilayers in amounts up to the maximum obtainable (50 mol %) and caused the partition coefficient of the undissociated molecule to fall, sharply at first, and then more slowly, whilst that of the anion was unaffected (Figure 3). Incorporation of up to 38 mol % of the negatively charged lipid, phosphatidic acid, into phosphatidylcholine bilayers also caused a reduction in the partition coefficient of the undissociated pentobarbitone.

The total ionic strength of the buffer solution was varied by changing the KCl concentration from 0 to 0.31M. At high pH the partition coefficient in

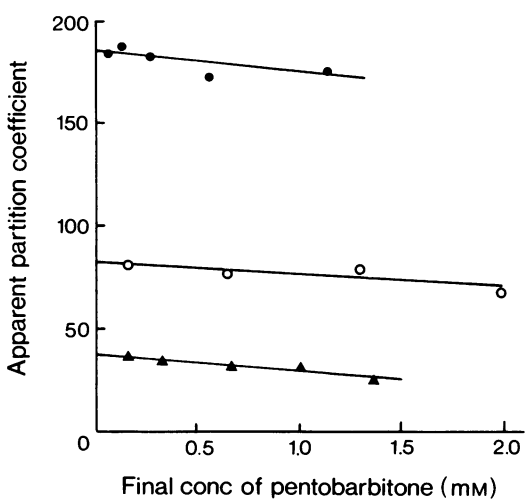

Figure 1 The dependence of the membrane/buffer partition coefficient at pH 6.95 upon the concentration of free pentobarbitone in the buffer. The partition coefficients in this figure and Figure 2 are expressed per g of phospholipid, not total lipid. (●) Phosphatidylcholine; (○) 72% phosphatidylcholine, 28% cholesterol; (△) 50% phosphatidylcholine, 50% cholesterol. Proportions of lipids are expressed on a molar basis.

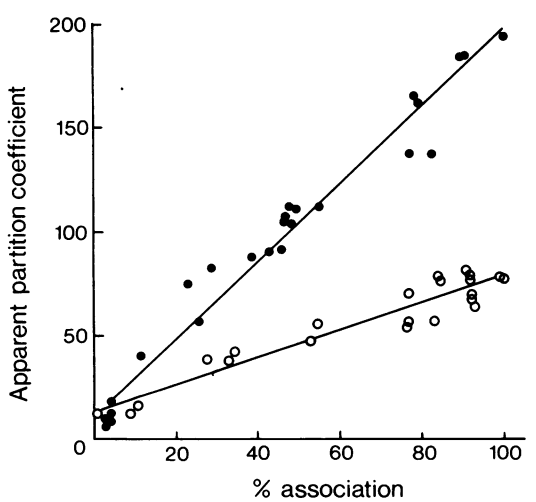

Figure 2 The dependence of the membrane/buffer partition coefficient (per g of phospholipid) on the degree of association of pentobarbituric acid (pKa = 8.0). (●) Phosphatidylcholine; (○) 75% phosphatidylcholine, 25% cholesterol.

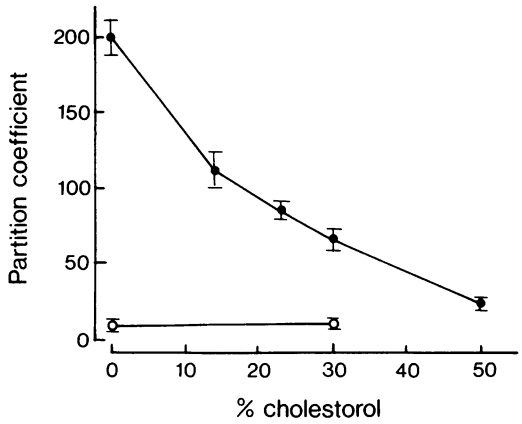

Figure 3 The dependence of the partition coefficient of pentobarbitone on the cholesterol content of phosphatidylcholine bilayer membranes. Partition coefficients are per g of total lipid (phosphatidylcholine + cholesterol). (●) Low pH; (○) high pH.

phosphatidylcholine bilayers was not markedly dependent on ionic strength, but the partition coefficient of the anion is apparently reduced to zero at high ionic strength. In contrast, at low pH a small decrease was observed at low ionic strength in phosphatidic acid-containing membranes (Figure 5).

Experiments with dialysis bags in the pressure bombs gave identical results to those obtained normally, but the standard deviations were much higher (between 10 and 20% of the mean). In three experiments at pH 7.8 the ratio of partition coefficient at one atmosphere to that at 400 atm was 1.13 for

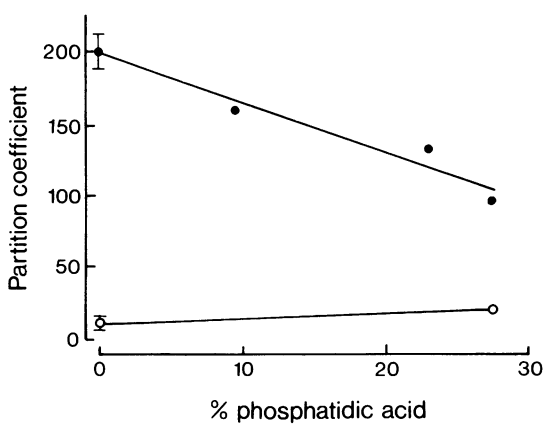

Figure 4 The dependence of the partition coefficient of pentobarbitone on the phosphatidic acid content of phosphatidylcholine bilayer membranes. (●) Low pH; (○) high pH.

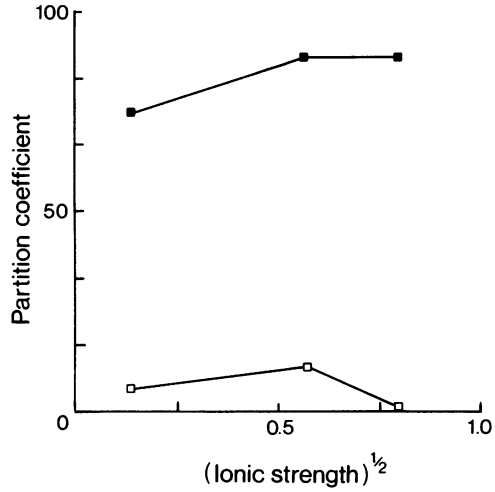

Figure 5 The effect of ionic strength. The relation between partition coefficient and the square route of ionic strength for (■) 30% phosphatidic acid, 70% phosphatidylcholine membranes at pH 4.0; (□) phosphatidylcholine membranes at pH 9.6.

phosphatidylcholine and 0.99 for phosphatidylcholine containing 30% cholesterol. No significant effect of pressure was observed therefore.

Discussion

The filtration technique gave values comparable to those we obtained by the conventional centrifugation technique. The main advantages of the method are the ease of equilibration with the monolamellar compared to the multilamellar vesicles, particularly with charged solutes, and the closer control of temperature possible during filtration compared to centrifugation. One drawback is the tendency for lipid, when present in high concentrations, to clog the filter. We also found that the combination of charged lipids and charged solutes can cause problems, particularly at low ionic strength, which probably result from an interaction between absorbed lipid and solute anion during passage through the filter.

One interesting observation from the control experiments was that the multilamellar vesicles consistently gave slightly lower partition coefficients than monolamellar ones. This was not an experimental artifact arising from the greater difficulty of obtaining equilibration in the multilamellar vesicles because the effect was observed with both the uncharged and the charged solutes (which are presumably less permeable and thus slower to equilibrate). A possible explanation is that the more fluid structure of the highly curved monolamellar vesicles (diameters in the range of

20–55 nanometres: Johnson, 1973) provides a better solvent for the barbiturate because less work has to be done to introduce the barbiturate between the lipid molecules.

The finding that the partition coefficient is independent of pentobarbitone concentration (Figure 1) is consistent with that obtained by Roth & Seeman (1972) in erythrocyte ghosts. This implies either that there are no free discrete binding sites at these concentrations (i.e. the membrane behaves as a homogeneous solvent), or that there are a very large number of such sites so that only a small fraction are saturated in the concentration range studied. The limited solubility of pentobarbitone prevents studies at significantly higher concentrations. The conclusions apply essentially to the uncharged solute since the data in Figure 1 are at pH 6.95 (pK of pentobarbitone is 8.0). Because the partition coefficient of the anion was not large compared to our errors it was not studied over a wide concentration range.

The effect of ionic strength at low pH (Figure 5) was similar in form to that reported for erythrocyte ghosts (Roth & Seeman, 1972). The binding of the anion is almost completely displaced by high ionic strength suggesting that surface electrostatic forces, perhaps between the anion and the charged choline group of the lipid (Blaustein & Goldman, 1966), predominate and that the anion does not significantly penetrate the bilayer. The A^-:phosphatidylcholine ratio is about 1:100 at 1 mM barbiturate and 0.16 M salt. At higher concentrations, charge-charge repulsion would limit the binding.

The effects of cholesterol and phosphatidic acid on the partition coefficient are illustrated in Figures 3 and 4 respectively. The marked decrease in partition coefficient for the uncharged barbiturate with increasing cholesterol content is similar to, but more pronounced than, that reported for benzyl alcohol (Colley & Metcalfe, 1972) and suggests that this may be a general phenomenon for small molecules. This is supported by the observation that incorporation of cholesterol into phospholipid bilayers reduced their permeability to small molecules (de Geir, Mandersloot & van Deenan, 1969).

There were no gross effects of cholesterol on the partition coefficient of the anion, though small trends would not be apparent with the level of precision obtained in these measurements because the observed coefficient was so small.

The effect of incorporating a negatively charged lipid, phosphatidic acid, which has been prepared from phosphatidylcholine from the same source as that used here and thus has the same acyl composition, is roughly parallel to that of cholesterol. Such an effect has apparently not been described previously.

The origin of the effects of cholesterol and phosphatidic acid on the phosphatidylcholine-buffer partition coefficient cannot be directly deduced from work of this nature. That would require a detailed spectroscopic study. However, it seems probable that a molecule with the amphiphilic characteristics of a barbiturate would locate primarily with the pyrimidine ring in the polar interfacial region of the membrane and the 5-alkyl substituents in the apolar interior, whilst a smaller proportion of molecules might be enclosed entirely within the apolar region. Nuclear magnetic resonance studies have been interpreted to support such a model for benzyl alcohol (Colley & Metcalfe, 1972).

The anaesthetic effect of pentobarbitone is reversible by pressure (Miller, 1972). Within the framework of the membrane hypothesis of anaesthetic action, this might arise because pressure displaced the anaesthetic from its site of action, or because pressure opposed the anaesthetic-induced membrane expansion. Mullins (1954) showed that the former possibility could not be ruled out, although it subsequently has been for gaseous anaesthetics (Miller, Paton, Smith & Smith, 1973). Our demonstration that high pressures do not appreciably alter pentobarbitone's partition coefficient is not consistent with the displacement model. Since the variation of partition coefficient with pressure reflects the difference between the partial molar volume of pentobarbitone in lipid and buffer, we may also conclude that this difference is small.

Our findings, if they can be extrapolated to biomembranes, raise an interesting question of specificity for the lipid theory of anaesthetic action. Neuronal membranes contain high proportions of cholesterol and charged lipids (Whittaker, 1969) and thus will tend to harbour lower concentrations of anaesthetic than other membranes such as mitochondria. In the case of pentobarbitone the observation that only membranes relatively high in cholesterol are fluidized may go some way towards answering this question, but for other anaesthetics this explanation is not possible (Miller & Pang, 1976).

How far may the lipid bilayer partition coefficients measured here be used to predict the values for biological membranes? The lipids of erythrocyte ghosts are 45 mol % cholesterol, and 17% of the phospholipids are negatively charged. The neutral phospholipids are phosphatidylcholine, phosphatidylethanolamine and sphingomyelin in roughly equal amounts. Phosphatidylserine is the major negatively charged lipid (Guidotti, 1972). If we segregate the lipids into three classes, neutral phospholipids, negatively charged phospholipids and cholesterol, and make the assumptions that these classes may be represented by phosphatidylcholine, phosphatidic acid and cholesterol, and, further, that the effects of these lipids are additive, we may use our lipid bilayer data to make a prediction of the partition coefficient in the erythrocyte ghost. Thus from Figure 4, λ is 138 when the negative lipid is 17%. Figure 3

Table 1 A comparison of partition coefficients measured in lipid bilayers, erythrocytes and bulk solvents

Partition coefficient in:	*Pentobarbitone*	*Benzyl alcohol*
Phosphatidylcholine bilayers	200 a	18 b
Phosphatidylcholine : cholesterol (1:1) bilayers	21 a	7 b
Erythrocytes	11 c,d	4 c
Octanol	89 e	13 e
Carbon tetrachloride	0.9 f	—

(a) This work; (b) Colley & Metcalfe, 1972; (c) Seeman, 1972; (d) Korten & Miller, 1976; (e) Hanch & Anderson, 1967; (f) Kakemi *et al.*, 1967.

shows the ratio of λ for phosphatidylcholine to that for 45% cholesterol in phosphatidylcholine is 0.17, which, under the above assumptions, yields an estimated λ for the phosphatidylcholine : cholesterol : phosphatidic acid bilayer of 23. The partition coefficient of red blood cell ghosts has been measured at low pH; values are 9.6 (Roth & Seeman, 1972) and 12 (Korten & Miller, 1976). Since only 44% by weight of the erythrocyte ghost membrane is lipid, and we further assume that negligible amounts of pentobarbitone are bound to the protein, then these values become 22 and 27 for the partition coefficient in the lipid bilayer regions of the erythrocyte ghost membrane. Such good agreement with our estimate based on the lipid bilayer is probably partly fortuitous, because the assumptions we have made are extremely crude; for example, the lipids in the red blood cell are not homogeneously distributed (Bretcher, 1973; Gordesky & Marinetti, 1973). Nevertheless the little systematic data available (Table 1) suggests that the lipid bilayer approach offers much better estimates of the absolute value of membrane partition coefficients than an approach based on model solvents. We propose that knowledge of a few readily measured lipid bilayer partition coefficients for a given solute should provide a relatively reliable means for predicting its partition coefficient in any biomembrane, or region of a biomembrane, whose composition is known. This procedure is more rational than that based on the use of model solvents, such as octanol (Leo, Hanch & Elkins, 1971), and deserves further investigation.

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