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MEMBRANE COMPOSITION MODULATES THIOPENTAL PARTITIONING IN BILAYERS AND BIOMEMBRANES

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Summary

The nonspecific interaction of thiopental with erythrocyte ghosts, synaptic membranes, microsomes and mitochondria has been measured at 25°C and pH 6.6. In cholesterol-depleted erythrocyte ghosts the partition coefficient decreases with increasing cholesterol content. In sonicated liposomes made from egg lecithin and cholesterol the partition coefficient also decreases with increasing cholesterol content. The dependence of the partition coefficient on cholesterol content in the biological membranes, on average, parallels that in the lipid bilayers. The partition coefficient in lipid bilayers made from lipids extracted from erythrocyte ghosts was comparable to that in the corresponding egg lecithin/cholesterol bilayer. The partition coefficients of all the biomembranes are consistently lower than those in the corresponding egg lecithin/cholesterol bilayer, the free energy of transfer between biomembrane and corresponding bilayer being −1 kcal/mol.

Introduction

The partitioning of hydrophobic solutes into biomembranes is of interest from several viewpoints. Thus, the free energy of partitioning into a membrane will provide some information about its average state on a time scale longer than that associated with most spectroscopic techniques. Whilst at a more practical level, both the passive diffusion of such solutes across cellular membranes and the pharmacological properties of some of them may be related to their membrane solubility [1,2]. It is thus of some interest to examine the variables which may influence such partitioning. In our studies of general anesthetics we have previously shown that, in lipid bilayers, increasing choles-

terol content lowers the partition coefficient [3], and we were interested in determining whether this effect was retained in biomembranes. The only evidence that this might be the case comes from a study showing that chlorpromazine partitioning into synaptic membranes is increased upon cholesterol depletion [4]. However, the latter data were interpreted as showing that the major proportion of the drug is associated with protein or phospholipid since their proportions increase upon cholesterol depletion [4]. On the other hand, we found that the partitioning of *n*-butane into erythrocyte ghosts is 4-fold less than into lipid bilayers [5], suggesting that the solute is mainly associated with the lipid region and that the ordering effect of proteins on lipid bilayer structure [6] might explain the decrease in partitioning. In order to examine the influence of membrane composition more systematically we chose to use a barbiturate as a solute because, of all the anesthetics we have examined, these show the greatest composition-dependence. Thiopental has a relatively high partition coefficient [2,7] and was consequently used in this study.

Given the heterogenous distribution of lipids and proteins in biomembranes [8], the difficulty in providing unequivocal answers to these problems is obvious. Nonetheless, measurements of an average property such as the partition coefficient might be expected to reveal the influence of some dominant variables. The parallels between lipid bilayers and biomembranes which our results yield do provide a self-consistent picture in which membrane composition plays an important role in modulating the partition coefficient of thiopental.

Methods

Membrane preparations

Erythrocyte ghosts were prepared by osmotic hemolysis from a recently outdated bank blood kindly supplied by J.W. Darnell. The hemoglobin-free red cell ghosts obtained after multiple centrifugations and washings [9] were resuspended in NaCl (0.9%, w/v) and either 6.25 mM phosphate buffer or 10 mM Tris-HCl (pH 6.6). 1 mM EDTA was added to all buffers. All biological material was kept at 4°C prior to its use.

Erythrocyte ghosts partially depleted of cholesterol [10] were prepared by incubation with sonicated egg lecithin vesicles. 2 g lipid dispersed in 80 ml of 0.1 M Tris-HCl buffer (pH 7.4) in isotonic NaCl were added to 70 ml of packed human erythrocyte ghosts, and incubation was carried out with gentle stirring for 15—20 h at 37°C in 700 ml buffer, NaN_3 (0.1%, w/w). Control ghost suspensions were treated similarly but no liposomes were added.

Synaptic membranes were prepared from frozen rat brains (Rockland, Gilbertsville, PA) which were defrosted and homogenized in 15 vols. of 0.32 M sucrose [11]. The supernatant remaining after centrifugation at $1000 \times g$ for 10 min was centrifuged at $20\,000 \times g$ for 20 min. The pellet was resuspended in distilled water, sonicated briefly and centrifuged at $8000 \times g$ for 20 min. The supernatant and the white upper buffy layer of the pellet were centrifuged at $48\,000 \times g$ for 20 min. The last step was repeated and the final pellet resuspended in phosphate buffer.

Mitochondrial and microsomal membranes were prepared from the livers of

male Charles River rats (CD strain, 150—220 g) which were homogenized in 8 vols. of 0.32 M sucrose [12], centrifuged twice (at 250 and 100 × *g*) for 10 min and the supernatant centrifuged at 8000 × *g* to sediment the mitochondrial fraction. The microsomal fraction was prepared by pelleting the 15 000 × *g* supernatant at 100 000 × *g* for 60 min.

Lipid bilayer vesicles were preapred from appropriate mixtures of egg lecithin (Lipid Products, Nutfield, U.K., Grade 1) and cholesterol (Sigma, recrystallized from CH_3OH) in $CHCl_3$. After evaporation of the $CHCl_3$, 0.15 M KCl/10 mM Tris-HCl (pH 7.0) was added and the suspension was sonicated (Sonifier Cell Disrupter, Heat Systems W 185, Plainview NY) at 0°C under N_2 and centrifuged at 20 000 × *g* for 20 min.

Lipid vesicles from red blood cell lipids were prepared similarly. These lipids were extracted using a modification of the procedure of Rose and Oklander [13]. 4.5-ml fractions of packed erythrocyte ghosts were incubated at 0°C in 100 ml isopropanol. 70 ml $CHCl_3$ were added and after standing the suspension was concentrated and centrifuged for 10 min at 650 × *g*. The supernatant was concentrated and centrifuged at 650 × *g* for 1 h. This procedure was repeated on the two sediments and the supernatants combined and evaporated to dryness.

Measurement of partition coefficient

Approx. 1 μCi of [^{35}S]thiopental (5-ethyl-5(1-methylbutyl)-2-thiobarbiturate (Amersham/Searle, Arlington Heights, IL; 10.5 Ci/mol initial specific activity, yielding a single spot by thin-layer chromatography)) was dissolved in buffer containing unlabelled thiopental. Weighed aliquots of barbiturate solution were added to 1 ml of membrane suspension (pH 6.6) containing 10—150 mg membrane and were stirred for 1 h at 25.0 ± 0.05°C in a water bath. Identical samples without [^{35}S]thiopental were treated similarly. The suspensions were then centrifuged at 12 000 × *g* for 30 min at room temperature (microsomal membranes, 100 000 × *g* for 45 min). Weighed aliquots of supernatant (approx. 0.1 g) and 20—100 mg of pellet, digested in 0.4 ml Protosol (New England Nuclear, Boston, MA) and 0.3 ml ethanol at 55°C and then decolorized with 0.1 ml 30% H_2O_2 for 30 min at 60°C, were counted in 10 ml Biofluor (New England Nuclear). Quenching was kept constant by including the blank supernatant with the radioactive pellet and vice versa. Samples in triplicate were counted to a 1% standard deviation. Other weighed aliquots of supernatant and pellet were dried overnight at 85°C in order to determine the dry weight of membranes.

Partition coefficients of sonicated lipid suspensions were measured by ultrafiltration through an Amicon XM-50 filter as previously described [3].

Partition coefficients were calculated as (mol thiopental/g dry membrane)/(mol thiopental/g buffer). Protein concentrations were determined by a modification [14] of the method of Lowry et al. [33] using bovine serum albumin (Sigma) as standard, phosphate by the method of McClare [15] and cholesterol by the method of Rudel and Morris [16].

Results

Barbiturates are weak acids and thiopental has a p*K* of 7.6 [17]. We have previously reported the pH-dependence of several barbiturates in erythrocyte

ghosts [7]. Essentially no partitioning was seen at high pH values and the non-charged form partitioned independently of pH. All partition coefficients reported here were determined at pH 6.6 to avoid exposing the biomembranes to excessively unphysiological conditions.

Partitioning was measured over a wide range of thiopental concentrations for erythrocyte ghosts and microsomal membranes. In erythrocyte ghosts, thiopental showed highly linear binding over the range 25—370 μM with a slope of 24 ± 1.2 (S.D.). The mean of this and all other experiments was 24 ± 3.8. This lack of saturable binding is also consistent with some previous work on pentobarbital [18]. On the other hand, microsomal membranes showed a small amount of saturable binding in the region 6—100 μM. From 0.1 to 0.9 mM the binding curve was highly linear. In one experiment the slope in the latter region yielded a partition coefficient of 75 ± 1.8. Few previous values of the binding of barbiturates to microsomes appear to have been recorded. Sitar and Mannering [19] reported linear binding curves in the range 0.1—1.0 mM for a number of barbiturates. Analysis of their data for the uncharged forms of phenobarbital and pentobarbtial yields partition coefficient values of 30 and 40, respectively, which when compared to our results for erythrocytes for all three barbiturates [7] suggests that our value for thiopental in microsomes to be essentially correct. Their results were insufficiently detailed for detecting saturable binding. Although it was not our objective to examine saturable binding in any more detail than that sufficient to correct the partition coefficient, it seems worth recording the results of Scatchard analysis which yielded an approximate figure of 2 mol thiopental/mg dry membrane and a dissociation constant of 20 μM for these sites. This affinity is within the range found by Bickel and Steele [20] for the binding of a wide range of compounds to rat liver microsomes.

Eight separate depletions of erythrocyte ghost cholesterol were carried out. Cholesterol and phosphate were determined in each case and gave on average 30 ± 2.3 mol% cholesterol compared to 43 mol% for the controls, a mean depletion of 30%. The depleted ghosts gave a partition coefficient of 37 ± 8.3 compared to a value for their matched controls of 27 ± 6.1. These values are significantly different according to the Student's t-test ($p = 0.014$). In a previous study, Seeman and Lee [4] depleted synaptosomal membranes with cholesterol-depleted plasma lipoproteins. The mean increase they observed in chlorpromazine's partition coefficient was 312 ± 218%, a value of the same sign but rather larger than that we obtained for thiopental. Their degree of cholesterol depletion was not reported.

For mitochondrial and synaptic membranes, partition coefficients of 50 ± 7.6 and 32 ± 0.5, respectively, were found. Thus, the partition coefficients in all these biomembranes only show a 3-fold variation which is quite remarkable considering the range of protein content and lipid composition they exhibit.

The extracted erythrocyte ghost lipids had a partition coefficient of 146 ± 12. Their cholesterol content was determined to be 44 mol%. The ghost lipids exhibit a 5-fold higher partition coefficient than the intact membrane. Colley et al. [21] have reported a similar finding for benzyl alcohol although the effect was less marked.

We have previously shown that pentobarbital partitions equally into multi-

TABLE I

THIOPENTAL MEMBRANE/BUFFER PARTITION COEFFICIENT AT pH 6.6 AND 25°C

Membrane	Cholesterol/ (cholesterol + phosphate)	λ (± S.D.)
Lipid bilayers		
egg lecithin	0	437 ± 17
egg lecithin/cholesterol	0.15	327
egg lecithin/cholesterol	0.33	215
egg lecithin/cholesterol	0.50	126
ghost lipids	0.44	146 ± 12
Biomembranes		
rat liver mitochondria	0.084	50 ± 7.6
rat liver microsomes	0.18 ± 0.053	75 ± 7.8
cholesterol-depleted erythrocyte ghosts	0.30 ± 0.023	37 ± 8.3
human erythrocyte ghosts	0.41 ± 0.057	24 ± 3.8
rat brain synaptic membranes	0.46	32 ± 0.5

and unilamellar lipid vesicles [3], so that the difference in curvature between sonicated vesicles and biomembranes is unlikely to be reflected in their partition coefficients.

The partition coefficient of thiopental in egg lecithin bilayers was 437, much higher than that in biomembranes. This value fell linearly with the cholesterol content of the bilayer, reaching a value of 126 at 50 mol% cholesterol. The effect is similar, though more highly linear, to that previously noted for pentobarbital [3]. We have no explanation for the slight difference in linearity.

Results of cholesterol assays are given in Table I. These are generally in agreement with literature values [22,23]. The value for microsomes was somewhat variable, probably reflecting the heterogeneity of this fraction [24]. Depletion of erythrocyte cholesterol was fairly consistent under controlled conditions, although occasionally depletion failed to occur and the partition coefficient then remained unchanged. The mean weight percent of protein in our membrane samples was: mitochondria, 67; microsomes, 51; erythrocyte ghosts, 57; synaptic membranes, 36.

Discussion

The factors which control the non-specific partitioning of lipid-soluble solutes into biological membranes are largely unknown. The heterogeneous composition of these membranes and the non-random organization of their components [8,23] make a detailed description of their solvent properties a formidable task. Nonetheless, by exploiting knowledge gained from well-defined lipid bilayers it should be possible to define at least the dominant variables at work in biomembranes. Several studies of partitioning into lipid bilayers have been made. They have demonstrated that among the variables determining the magnitude of partitioning into phospholipid bilayers are the physical state of the lipid, the degree of unsaturation of the acyl chains, the surface charge and the cholesterol content [3,5,25,26].

Our data with thiopental reaffirm the importance of cholesterol in phospho-

lipid bilayers; we found that there was a 7% decrease in partition coefficient per mol% increase in cholesterol content. A similar effect amounting to 12% was observed over a narrower range of cholesterol content when erythrocyte ghosts were compared with cholesterol-depeleted ghosts. These observations suggest that cholesterol modulates thiopental partitioning into both lipid bilayers and erythrocyte ghosts.

In addition, the presence of protein in erythrocytes decreased the partitioning of thiopental 6-fold below that found in the extracted lipids. Since our partition coefficients are expressed per g of membrane, some of this difference might arise because the contribution of erythrocyte protein to partitioning is very much less than that of lipid on a weight basis. This problem is compounded by the heterogeneity of the ghost protein, much of which is peripheral to the membrane [8,23,24]. However, even when we arbitrarily assign all the thiopental to the lipid region of the erythrocyte membrane we only obtain an upper limit to the partitioning per g of membrane lipid of 56, or some 2.6 times less than that in the extracted lipids. A further interesting point is that egg lecithin/cholesterol bilayers with the same mole percent of cholesterol as the extracted erythrocyte lipids have an interpolated partition coefficient of 154 ± 20 compared to that of 146 ± 12 for the erythrocyte lipids themselves. This remarkable coincidence is probably partly fortuitous because erythrocyte phospholipid contains phosphatidylcholine, phosphatidylethanolamine and sphingomyelin in roughly equal proportions, together with a lesser amount of phosphatidylserine [22]. On the other hand, the head groups of these lipids may not influence thiopental partitioning strongly. Thus, in one experiment we found that, although adding phosphatidylserine to phosphatidylcholine bilayers caused a linear decrease in the partition coefficient, even in pure phosphatidylserine bilayers the reduction was only 34%.

Before considering how far these conclusions are valid when a wider group of biomembranes are examined, our results must be put on a thermodynamic basis. Thus, we define the standard free energy of solution as $\Delta G^0 = -RT \cdot \ln \lambda_0$,

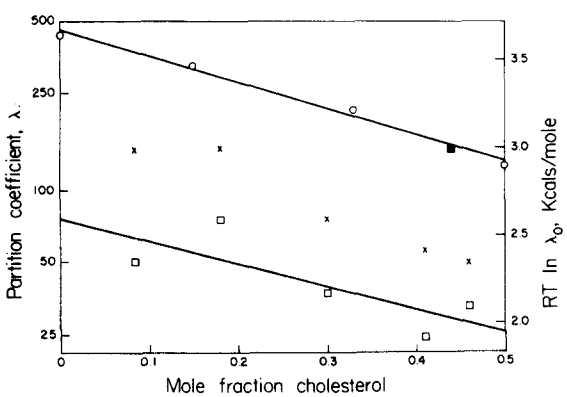

Fig. 1. The partition coefficient of thiopental as a function of the mole percent of cholesterol in the lipids of biomembranes (□), egg lecithin/cholesterol bilayers (○) and extracted ghost lipids (■). When the biomembrane partition coefficients are expressed per g of membrane lipid instead of per g of membrane, an upper limit for the partitioning of thiopental into membrane lipid is obtained (X).

where R is the gas constant, T the absolute temperature and λ_0 the partition coefficient for the completely associated barbiturate. The data from Table I are presented in this way in Fig. 1 where they are plotted against the mole fraction of cholesterol in total lipid.

The free energy of transfer of thiopental from buffer into egg lecithin bilayers increases with cholesterol content in a highly linear manner. The slope of the least-squares line through these points is 1.5 ± 0.11 kcal/mol per mole fraction cholesterol. A similar trend is seen when the biomembranes are considered: the slope is 1.3 ± 0.55 kcal/mol per mole fraction cholesterol. In this case the fit is much poorer. This is to be expected since cholesterol is not the only variable influencing partitioning into this heterogeneous group of membranes. Although we cannot rule out that these slopes correspond by chance, it seems reasonable to conclude, tentatively, that among several operational variables the effect of cholesterol is strong enough to produce the overall trend observed. This conclusion is supported by the results with cholesterol-depeleted ghosts.

Fig. 1 also shows that all the biomembranes fall below the line for the corresponding egg lecithin/cholesterol bilayer, even when we make the limiting assumption that all the thiopental is in the biomembrane's lipid bilayers. We prepared insufficient quantities of membranes to perform experiments on extracted lipids in all cases, but there is no major class of phospholipid present in these membranes that does not occur in erythrocytes and, except for diphosphatidylglycerol, in mitochondria. Thus, it seems probable that in all cases the presence of membrane protein is responsible for decreasing the partitioning of thiopental. In the case of erythrocytes our data show that the free energy change between each biomembrane and its equivalent egg lecithin/cholesterol bilayer is −1.0 ± 0.16 kcal/mol. Even when the biomembrane partition coefficients are expressed per g of lipid an average free energy change of −0.6 ± 0.10 kcal/mol remains to be explained.

The origin of the similar effects of cholesterol and protein must be sought by molecular methods. However, it is well known that cholesterol increases the anisotropy of packing in lipid bilayers [27,28]. Similarly, several studies on reconstituted membranes show that proteins, such as rhodopsin [6] and cytochrome *c* oxidase [29], can cause fairly extensive ordering in lipid bilayers. Thus, a plausible hypothesis is that this closer packing of the bilayer, which would presumably result in stronger van der Waals interactions between the acyl chains of the lipids, requires the expenditure of additional free energy in order that a molecule of thiopental may be inserted into the bilayer.

This explanation assumes that the membrane-anesthetic interaction involves simple bulk partitioning. In a solvent only two molecules thick, interactions, which may be realtively specific [30], might occur between lipid head-groups and solutes. If so, there must be a large number of such sites with low affinity leading to a description quantitatively indistinguishable from bulk partitioning. Although an explanation for our results based on protein- and cholesterol-induced modifications in the head-group regions cannot be ruled out, it is made less likely by results showing that the partitioning of the much less polar solute, halothane, has a similar composition-dependence (Smith, R.A. and Miller, K.W., unpublished data, and Ref. 32).

In addition to the above considerations our results are also of some relevance to the mechanism of action of general anesthetics. These agents are thought to block excitable membranes either by binding directly to a non-polar region of a protein or by perturbing its surrounding lipids [2]. Although our data show that anesthetic partitioning decreases with an increasing membrane protein content, this cannot be used as an argument against the direct protein binding hypothesis because a single binding site in an excitable membrane could easily remain undetected [32]. Our results do, however, pose a problem for the lipid hypothesis because synaptic membranes have high contents of both protein and cholesterol. Thus, the most probable putative site of action of anesthetics will tend to have the lowest membrane concentration of anesthetic. Such a criticism might be rebutted on the grounds that such excitable proteins are very sensitive to lipid perturbations, or that their boundary lipids are more readily perturbed than bulk lipid or that the latter are not of average lipid composition. Only detailed studies of excitable membranes can answer the problem posed by this work.

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