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DIPHENYLHYDANTOIN BINDING TO BRAIN LIPIDS AND PHOSPHOLIPIDS

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Abstract— Partition coefficients for diphenylhydantoin (DPH) were determined using four organic solvents and a 0.1 M phosphate buffer. Values ranged from 25.5 for chloroform to 0.02 for hexane. When chloroform–methanol-soluble brain lipids were added, there was a marked enhancement of DPH entry into hexane; 2 mg lipid resulted in an almost equal distribution between hexane and buffer. Proteolipids produced a similar but quantitatively small change. Neutral fat, cholesterol and glycolipids had no effect on DPH distribution. A number of commercially available phospholipids were tested and all increased the hexane/aqueous partition coefficient of DPH, although there was considerable variation among the several phospholipids employed. Neither total DPH concentration nor the addition of cations influenced this distribution. These results provide strong evidence for binding of DPH to phospholipids.

Many studies have demonstrated effects of diphenylhydantoin (DPH) on a wide range of biological systems, and a number of explanations of its actions have been proposed. Although there is no agreement on the precise mechanisms involved, the general effect of membrane stabilization is widely recognized. This concept implies interaction of diphenylhydantoin with one or more membrane components, the chemical compositions of which are unknown. Previous studies in this and other laboratories [1–3] have demonstrated that DPH is bound to a number of brain subcellular fractions as well as to other tissues. We have demonstrated that binding correlates very strongly with protein content, regardless of the fraction or tissue of origin [1, 4]. Furthermore, removal of lipid by extraction with acetone or chloroform–methanol enhances tissue protein binding [4]. Conversely, enzymatic hydrolysis of protein reduces binding capacity. Protein binding is not influenced by DPH concentration, temperature or cation concentration, and is relatively non-specific [4].

The above studies were all carried out in aqueous media which would favor hydrophilic binding and might obscure any hydrophobic interaction. In view of the known lipid solubility of DPH and most other depressant drugs [5], it appears likely that such a lipid interaction occurs. Seeman [5] has pointed out that there is excellent correlation between aqueous/non-aqueous partition coefficients and the potency of a number of drugs. Modifications of the partition coefficient technique have been useful in the study of local anesthetics [6], the cholinergic receptor [7], and opiate receptors [8]. We have investigated the interaction of DPH with brain lipids using similar techniques and report evidence for binding of DPH by brain lipids and the differential binding of DPH by phospholipids.

METHODS

Materials. Diphenylhydantoin (phenyl[4-^{3}H]) (47.5 Ci/m-mole) was obtained from New England Nuclear and diphenylhydantoin[4-^{14}C] (5.5 mCi/m-mole) was obtained from Schwarz–Mann. Radiochemical purity was established by ascending thin-layer chromatography in two solvent systems as previously described [1]. Gangliosides from bovine brain, cerebrosides from bovine brain, L-α phosphatidylethanolamine from ovine brain, and cholesterol were obtained from Sigma Chemical. Sphingomyelin from bovine brain, L-α lecithin (β, γ dipalmitoyl, synthetic) and L-α lecithin (dilauryl) were obtained from Cal-Biochem. Phosphatidyl-L-serine was obtained from Schwarz–Mann. Hexanes, a mixture containing primarily *n*-hexane, was obtained from Mallincrodt and was of analytical reagent quality, as were all of the other chemicals used.

Lipid extraction. Lipid extraction was carried out using the method of Folch *et al.* [9]. New Zealand white rabbits weighing 1.5 to 2.0 kg were sacrificed by decapitation and their brains quickly removed, weighed and placed in 10 vol. of ice-cold chloroform–methanol (2:1). The tissue was homogenized in a Virtis S-45 blender at high speed for about 1 min until a uniform suspension was obtained. The homogenate was centrifuged at 8000 *g* for 5 min and the supernatant washed with 0.2 vol. of distilled water. The aqueous layer was removed and discarded and the chloroform layer evaporated *in vacuo* to dryness. The resulting fraction, referred to as total brain lipids, was weighed, redissolved in chloroform and filtered through Wattman 1 PS filter paper. Subcutaneous fat was obtained from the abdominal region or rabbits and subjected to the above extraction to obtain the lipid fraction. Synaptosomes and myelin were isolated from rabbit brain as previously described [10] and extracted in an identical fashion to obtain synaptosomal lipid.

To obtain proteolipids, the chloroform fraction from the original extraction was mixed with 4 vol. of cold ether and the resulting proteolipid precipitate removed by centrifugation for 15 min at 2000 *g*. It was dried *in vacuo*, weighed and redissolved in chloro-

form–methanol. The protein content of this material was between 175 and 200 μg/mg when determined by the method of Lees and Paxman [11]. The commercially obtained purified lipids were weighed and dissolved in chloroform–methanol (2:1) except cerebrosides, which were dissolved in chloroform–methanol–water (10:10:3). Aliquots of each lipid solution were placed in tared beakers, the solvent was evaporated, and the weight/ml determined. All lipid values are expressed as dry weight.

Partition coefficients. Partition coefficients were determined using 2 ml of each of aqueous and non-aqueous phases. For most experiments, the aqueous phase was a 0.1 M sodium phosphate buffer (pH 7.0), which will be referred to as buffer throughout the text. In general, the appropriate quantity of lipid was added to a test tube and the solvent evaporated under nitrogen. The lipid was then dissolved in 100 μl chloroform–methanol and the non-aqueous phase added. The aqueous phase containing the radioactive DPH dissolved in ethanol was added last. Preliminary experiments revealed that maximum partitioning could be obtained with 30 sec of vigorous agitation on a Vortex mixer at 20–22° and this time period was used uniformly in the experiments reported here. After vortexing, the phases were allowed to separate by standing at room temperature. Occasionally centrifugation was necessary for complete separation. The upper phase was then removed with a Pasteur pipette and 0.1-ml samples of each phase were taken and placed in scintillation vials. Great care was taken to avoid any contamination during the sampling procedure. All experiments were done in duplicate and two samples of each obtained for counting. With two exceptions there was no interface or emulsion between the two phases. With dilauryl lecithin there was some opalescence at the interface which did not interfere with sampling. Cerebrosides, however, were insoluble in either phase and the solid cerebroside material was accumulated at the interface. Sampling of both phases was done as usual, the particulate matter was then centrifuged and dissolved, and samples were taken for counting.

Radioactivity determination. Each phase 0.1 to 0.2 ml was placed directly into scintillation vials and the organic solvents were allowed to evaporate in room air. Bray's solution [12] was then added and the vials were shaken vigorously and counted in a Beckman liquid scintillation counter at less than 3 per cent standard error. Quenching was determined in a number of samples by the addition of internal standard, no significant quenching occurred and, therefore, no corrections were necessary. Partition coefficients are expressed as the ratio of radioactivity in the non-aqueous phase to radioactivity in the aqueous phase.

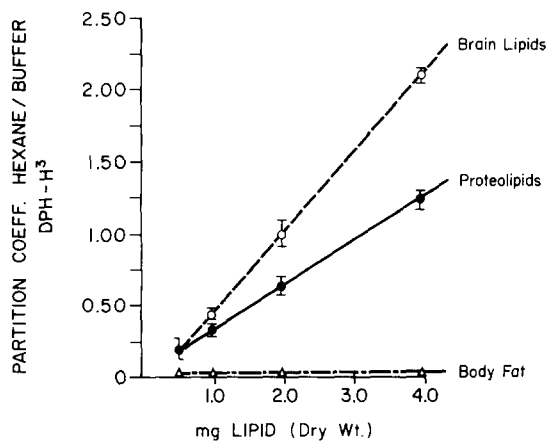

Fig. 1. Effect of lipid concentration on hexane–buffer partition coefficient of DPH[^{3}H]. Each point is the mean of at least six experiments. Brackets indicate standard deviation.

RESULTS

Brain lipids. In the initial experiments, the partition coefficient of DPH[^{14}C] was determined using four non-aqueous solvents (Table 1). Chloroform and dichlorethane showed a very high organic:aqueous ratio reflecting the marked solubility of this drug in agents with relatively high dielectric constants. Brain lipid did not significantly alter the partition of DPH into these solvents. When toluene was used (dielectric constant 2.38), the solubility of DPH was much less, and there was a small but definite enhancement of entry into the organic phase when brain lipids were added which increased with greater amounts of lipid. When hexane was employed as the organic phase (dielectric constant 1.89), there was virtually no entry of DPH into the organic phase, but with the addition of brain

Table 1. Effect of brain lipid on DPH[^{14}C] partition coefficients*

Organic phase	Brain lipids (mg)	P.C.†	Difference (%)
Chloroform		25.5	
Chloroform	1.0	25.3	NS‡
Dichlorethane		24.1	
Dichlorethane	1.0	24.8	NS
Toluene		4.3	+19
Toluene	1.0	5.1	
Hexane		0.02	+2000
Hexane	1.0	0.42	

* Each value represents the mean of three to six experiments.
† Partition coefficient organic phase vs 0.1 M PO_4 buffer (pH 7.0).
‡ No significant difference.

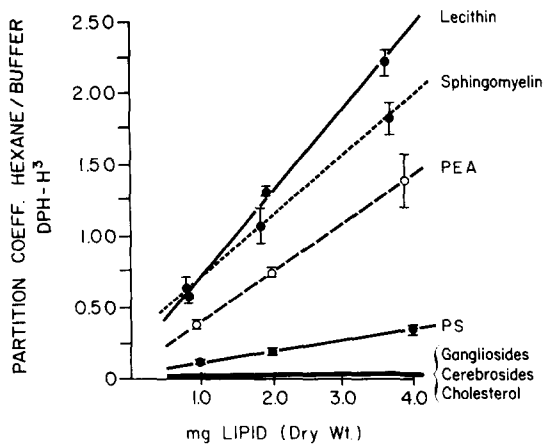

Fig. 2. Effects of commercially obtained, purified lipids on hexane DPH partition coefficients. Each point is the mean of six experiments. Brackets indicate standard deviation. PS = phosphatidyl-serine. PEA = phosphatidylethanolamine.

lipids there was a marked enhancement of DPH in the hexane phase. Hexane was then selected as the non-aqueous phase for the remainder of the studies.

The effect of brain lipid on the partition coefficient of DPH[^{14}C] into hexane is shown in Fig. 1. It can be seen that within the lipid concentrations used, the effect is significant and approximately linear. At higher lipid concentration, there is leveling out of the effect with a partition coefficient of 2.90 with 10 mg lipid. When proteolipids were used, there was again enhancement of DPH entry into the non-aqueous phase, but the effect was less pronounced than with the whole brain lipid fraction. Subcutaneous fat extract had no effect on DPH entry into the organic phase, and the results did not differ from the lipid-free control. The effect of lipids extracted from a synaptosome-enriched fraction was identical to the effects of whole brain lipids in enhancing the entry of DPH into hexane.

Phospholipids. In order to determine which fraction of the brain lipid extract was responsible for altering the partition coefficient of DPH, a series of commercially obtained lipid components of brain were tested individually for their effect on the entry of DPH[^{14}C] or DPH[^{3}H] into hexane. Figure 2 summarizes the results of these studies. It can be seen that all phosphorus-containing lipids had an effect on binding of DPH, with dipalmitoyl lecithin showing the greatest activity/mg. Dilauryl lecithin produced an opalescent interface which could not be sampled; however, the dilauryl derivative had less influence on the entry of DPH into hexane than did the dipalmitoyl compound (Table 2). Sphingomyelin had approximately the same action as whole brain lipid and phosphatidylethanolamine slightly less than whole brain lipids. When the *N,N*-dimethyl derivative of phosphatidylethanolamine was employed, the effect on the partition coefficient of DPH was identical to the non-methylated compound (Table 2). Phosphatidylserine, on the other hand, had relatively less binding activity/mg of lipid as compared to the other phospholipids.

Non-phosphorus-containing lipids, gangliosides, cholesterol and cerebrosides had no effect on the distribution of diphenylhydantoin between aqueous and non-aqueous phases and were equal to lipid-free controls. As noted previously, in the cerebroside experiment white particulate matter clumped at the interface and the radioactivity in this material was determined. A small percentage of total radioactivity accumulated in the cerebroside material which increased with increasing quantities; however, washing of this material with fresh buffer removed most of this activity.

In several experiments, the partition coefficient was determined after the addition of the anionic detergent sodium dodecyl sulfate to the buffer. With concentrations of up to 10 mg there was no alteration in the partition of DPH over control values. When 5 mg of whole brain as a homogenate, 5 mg of a myelin-enriched fraction or of a lipid-free protein fraction

Table 2. Comparative effects of lipids and non-lipids on DPH[^{3}H]partition between buffer and hexane*

	P.C. ± S. D.
Control (no addition)	0.02 ± 0.01
Brain lipid	0.39 ± 0.07
Proteolipid	0.33 ± 0.06
Phosphatidylethanolamine	0.39 ± 0.03
N,N-dimethyl phosphatidylethanolamine	0.36 ± 0.02
Phosphatidylserine	0.11 ± 0.01
Sphingomyelin	0.66 ± 0.05
Lecithin (dilauryl)	0.10 ± 0.01
Lecithin (dipalmitoyl)	0.66 ± 0.05
Sodium dodecyl sulfate	0.02
Whole brain (5 mg)	0.01
Myelin (5 mg)	0.08
Lipid-free protein	0.00
Olive oil	0.00

* Partition coefficients ± standard deviation with 1 mg of each material added unless otherwise specified. Where no S. D. is given, the result is the mean of three experiments; others represent the mean of at least six experiments.

Table 3. Effects of unlabeled DPH on partition coefficients of DPH[^{3}H]*

Total DPH concn. (M)	P.C. DPH[^{3}H] ± S. D.
0.5×10^{-12}	0.42 ± 0.05
1×10^{-10}	0.37 ± 0.03
1×10^{-8}	0.37 ± 0.03
1×10^{-7}	0.37 ± 0.05
1×10^{-6}	0.35 ± 0.02
1×10^{-5}	0.37 ± 0.01
1×10^{-4}	0.32 ± 0.03

* Each tube contained 0.25 pM DPH[^{3}H] plus the appropriate amount of unlabeled DPH. Each value is the mean of four experiments.

of brain were added and the usual partition experiment was carried out, none of these tissue fractions produced an alteration in DPH distribution when compared to control, tissue-free experiments. Olive oil was also tested with similar negative results on DPH partition (Table 2).

Concentration and ion effect. In order to study the effect of DPH concentration on binding to the lipid fraction, unlabeled DPH was added to the test system in various concentrations in a range of 0.5×10^{-12} to 1×10^{-4} M. Table 3 shows results of these experiments. Increasing or decreasing the total concentration of DPH present in the buffer did not appear to affect the partition coefficient of radioactive DPH, indicating no relationship to concentration. To assess the effect of various ions, calcium, potassium and magnesium in concentrations of 10 mM were added to the buffer and did not influence lipid-induced alterations in the partition coefficient (Table 4). The contribution of sodium ion in the phosphate buffer was evaluated by several experiments in which 0.1 M Tris–HCl buffer was employed. The results using this buffer in a sodium-free medium did not vary from the standard procedure using a phosphate buffer. The pH did influence the relative partition of diphenylhydantoin. When a pH of 5 or 6 was employed, the partition coefficients were approximately the same as with pH 7. However, with buffer of pH 8 there was a definite decline in the distribution of DPH into hexane and at a pH of 9 there was marked decrease of DPH concentration in the hexane phase. These values were still significantly higher than control lipid-free values.

Table 4. Effects of pH and cations on DPH[^{3}H] partition coefficients*

Aqueous phase	P.C.
Phosphate, pH 6	0.42
Phosphate, pH 7	0.38
Phosphate, pH 8	0.26
Tris buffer, pH 5	0.37
Tris buffer, pH 7	0.34
Tris buffer, pH 9	0.06
Phosphate, pH 7	
$+K^+$ 10 mM	0.32
$+Mg^{2+}$ 10 mM	0.34
$+CA^{2+}$ 10 mM	0.36

* Hexane is the non-aqueous phase in each case. Each value is the mean of at least three experiments.

DISCUSSION

This study demonstrates that brain lipids and a number of phospholipids are capable of solubilizing DPH in hexane, a compound with a low dielectric constant in which it is otherwise insoluble.

In the initial experiments using chloroform, no enhancement could be detected, presumably because of the high solubility in this solvent. The hexane system, therefore, was much more sensitive for detecting quantitative differences among the compounds tested. Lowney *et al.* [8] employed a similar procedure in their study of opiate binding. Subcutaneous fat and other neutral lipids, such as olive oil, had no effect on the distribution of DPH, indicating that there is some specificity in the brain lipid preparation.

Proteolipids appear to be involved in several specific drug receptors [7, 8], and we have previously shown significant binding of DPH to brain protein [1, 4]. However, when the proteolipid fraction was studied, relatively less binding was encountered than with the whole brain lipid fraction. This is not surprising since protein binding involves hydrophilic mechanisms, whereas the techniques employed in the present study minimizes hydrophilic attractions. The extent of binding by the proteolipid fraction probably reflects the lipid content of this fraction rather than a lack of a protein interaction.

Studies employing commercially obtained lipid fractions are somewhat limited because of the variety of sources of these materials and possible contamination with impurities. However, the consistency of the data obtained suggests that these factors are of minimal importance. All of the major lipid constituents of brain were investigated. Neither glycolipids nor cholesterol altered the partitioning of DPH, but all of the phospholipids investigated had definite binding properties. It is difficult to evaluate the significance of the quantitive differences among the phospholipids employed because of the variability in the fatty acid composition of each; however, our study demonstrates that such factors may be important. Dilauryl lecithin showed considerably less binding than the dipalmitoyl derivative. On the other hand, *N,N*-dimethyl phosphatidylethanolamine was equivalent to the non-methylated compound, indicating that masking of the amine group had little effect. Further studies are needed to determine the exact influence of fatty acid composition of phospholipids on DPH binding.

We were unable to alter DPH binding by changing the ionic conditions of the study. In this respect, diphenylhydantoin appears to differ considerably from local anesthetics in that bivalent cations inhibit the binding of procaine [6]. Pincus and Lee [13] and Carnay and Grundfest [14] have also suggested an important interaction of calcium with diphenylhydantoin at neuromembranes, but our studies were unable to correlate any direct effect of calcium on DPH binding to brain lipids or proteins [4]. Potassium, magnesium or sodium ion concentrations did not appear to play any role. At high pH the increased

solubility of DPH in the aqueous phase decreased the entry of this drug into hexane, although changes in the ionization of the lipids and their capacity to bind may also be involved. Furthermore, this binding appears to be independent of DPH concentration over a large concentration range. This finding is very similar to the non-saturable binding we have described in aqueous protein-containing media [4]. Ion-dependent binding and concentration-dependent binding for specific phospholipids may occur that are not apparent with the whole brain lipid fraction.

The nature of the interaction of DPH with phospholipids is not fully resolved by this investigation. One possibility is that the effect is due to non-specific surfactant properties of phospholipids. However, when the anionic detergent sodium dodecyl sulfate was employed it did not influence DPH entry into hexane. Cerebrosides have been reported to form complexes with several amines [15] and to bind morphine derivatives stereospecifically [16]. In this study, cerebrosides had no influence on the entry of DPH into hexane but were not completely solubilized, allowing sampling of cerebrosides independently. A small percentage of the radioactivity was associated with the lipid but most of it was easily removed by washing with fresh buffer, suggesting that there was contamination from the aqueous phase although minimal binding of DPH by cerebrosides may occur.

We tentatively conclude that the interaction of phospholipids with DPH does represent true binding perhaps with the formation of a DPH–phospholipid complex, but the exact nature of the bonds formed is unknown. This and previous studies from our laboratory have now demonstrated significant binding of DPH by the principal chemical constituents of cell membranes, proteins and phospholipids. The principal pharmacological action of the agent in usual therapeutic concentrations is to stabilize excitable membranes which have been rendered unstable in some way. This membrane action implies an effect of the drug on membrane constituents, and binding of both protein and phospholipid represents a mechanism for this effect.

Carnay and Grundfest [14] studied the action of DPH on the neuromuscular junction and concluded that DPH acts by producing conformational changes in the membrane as a result of hydrophobic binding. Pincus and Lee [13] have also postulated conformational changes resulting in decreased membrane conductance of cations. Seeman [5] has summarized a large body of data correlating the membrane effects of a number of anesthetic drugs with their ability to protect erythrocytes from hemolysis and to expand erythrocyte membranes and lipid monolayers at concentrations which block the membrane action potential. He has also pointed out that the aqueous/non-aqueous partition coefficient of these agents correlates extremely well with anesthetic activity. Blaustein and Goldman [6], in studies similar to the present investigation, report that several phospholipids enhanced the solubility of procaine and other local anesthetics in chloroform–methanol. Although several authors [5, 14] have noted similarities between DPH and local anesthetics in their membrane actions, there are obviously significant pharmacologic and therapeutic differences among these agents, and the failure of calcium to influence DPH binding is another important difference. Nevertheless the present study supports the concept of a general DPH–membrane interaction. The precise mechanism whereby this interaction results in a specific pharmacologic action by this drug is determined by the tissue affected and the nature of the destabilizing process.

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