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BINDING OF DIPHENYLHYDANTOIN TO BRAIN PROTEIN

MARK A. GOLDBERG and THEODORE TODOROFF

Departments of Pharmacology and Neurology, Harbor General Hospital Campus of UCLA, Torrance, Calif., U.S.A.

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Abstract—The binding of radioactive diphenylhydantoin (DPH-^{14}C) to brain homogenates and isolated subcellular fractions of brain has been evaluated. Homogenates of mouse brain diluted to various concentrations were incubated with DPH-^{14}C and subjected to an ultrafiltration procedure to evaluate tissue binding. The percentage of bound drug was related to the tissue concentration of the homogenate. Subcellular fractions of brain were isolated and incubated in a similar manner and DPH binding was directly related to protein content of each fraction. Fractions subjected to sonication bound DPH to the same extent as intact fractions. Studies *in vivo* and *in vitro* of DPH-^{14}C binding to brain which was subjected to subsequent fractionation revealed a differential distribution. There was no significant difference in the distribution pattern at 2 or 14 hr and the patterns *in vitro* were similar. It is concluded that DPH-^{14}C is reversibly bound to brain components and that binding capacity is directly related to protein content.

THE CORRELATION between blood levels of diphenylhydantoin (DPH) and clinical control of seizures is generaly recognized and the measurement of these levels has become routine in many clinics.[1] Binding of DPH to plasma proteins is known to play an important role in the distribution of this drug. In humans, approximately 90 per cent of blood diphenylhydantoin is bound to plasma protein,[2] and cerebrospinal fluid DPH is approximately equal to the concentration of the unbound plasma fraction.[2,3] Binding of DPH to brain has also been reported by several authors. Firemark *et al.*,[3] studying the distribution of ^{14}C-labeled DPH in the cat, found brain levels of this drug were 5–10 times higher than the levels in a plasma ultrafiltrate 30 min after intravenous injection. Approximately 90 per cent of the total DPH in cat brain was bound to some tissue constituent. These authors reported that all of the radioactivity in brain was in unmetabolized DPH. A similar finding has been reported by Noach *et al.*[4] Kemp and Woodbury,[5] in a preliminary report, suggested that there was differential binding to subcellular fractions of rat brain which varied with time. In their preliminary report and in a more detailed paper published recently,[6] they report an increasing percentage of radioactivity associated with the microsomal fraction over a period of 12 hr. They suggested that this binding was covalent and could only be released upon alkaline hydrolysis. Woodbury[7] has speculated, on the basis of these findings, that DPH may be incorporated into messenger RNA and thereby affect protein synthesis by the ribosomal system. A number of subsequent reports[8–11] employing differing methods of isolation of subcellular fractions have appeared which have resulted in conflicting evidence on the distribution of DPH.

We have investigated the subcellular binding of DPH-^{14}C in mouse and dog brain using differential centrifugation and the ultrafiltration procedure of Schanker and Morrison.[12] Utilizing the latter method and employing pre-isolated subcellular fractions minimize the problems of redistribution and trapping, and avoid strong centrifugal fields which affect binding. Our data indicate that DPH binding can best be related to protein content of brain fractions.

MATERIALS AND METHODS

Male, C57 Bl_6 mice obtained from Sprague–Dawley were used in most experiments. When larger quantities of neural tissue were required, mongrel dogs were used.

Diphenylhydantoin-4-^{14}C (4·47 mCi/m-mole) was obtained from Tracerlab and shown to be radiochemically pure by ascending thin-layer chromatography on Gelman type S medium using *n*-butanol–ammonium hydroxide (75:25) and autoradiography.

Cellulose dialysis tubing, $\frac{1}{4}$ inch diameter with an average pore size of 48 nm, was obtained from Arthur Thomas Co. All chemicals were of at least reagent grade.

Experiments in vivo. Solutions of DPH-^{14}C were prepared freshly by dissolving a weighed sample in a drop or two of 0·25 N sodium hydroxide and the appropriate volume of distilled water. Mice were given a dose of 25 mg/kg in 0·1 ml by the intraperitoneal route, and sacrificed by decapitation 2 or 14 hr later. The whole brain was quickly removed and placed in 10 vol. of ice-cold 0·32 M sucrose and homogenized at 465 rev/min by seven up-and-down strokes in a Thomas glass–Teflon homogenizer. The homogenate was centrifuged for 10 min at 1000 *g*. The resulting pellet was gently resuspended in 5 vol. of the cold sucrose solution and recentrifuged. The remaining pellet contained nuclei and cellular fragments.[13–15] The combined supernatants were centrifuged at 15,000 *g* for 30 min to obtain the crude mitochondrial fraction. The post-mitochondrial supernatant was centrifuged at 100,000 *g* for 60 min in a Spinco model L ultracentrifuge to obtain the microsomal pellet. In some experiments the crude mitochondrial fraction was subfractionated according to the method of Whittaker,[13] using a discontinuous gradient of 0·8 and 1·2 M sucrose and centrifuged for 60 min at 53,000 *g* in a swinging bucket rotor (SW-50·1). In this system myelin layers at the top of the 0·8 M sucrose, synaptosomes at the 0·8 to 1·2 M interface, and mitochondria form a pellet at the bottom of the tube.[13–15] These fractions were easily removed with a Pasteur pipette.

The particulate fractions were suspended in 1–2 ml sucrose solution, the exact volume was measured and aliquots were removed for protein and radioactivity determinations. The pH of brain homogenates and the subcellular fractions in 0·32 M sucrose was 6·7 to 6·8.

Experiments in vitro. The 10% homogenates, prepared as described above, were incubated with 1 μCi DPH-^{14}C in a shaking water bath at 37° for 15 min. Incubation was terminated by placing the tube in an ice bath and subfractions were obtained as above, using 0·32 M sucrose for dilution. To study the binding properties of isolated subcellular fractions, larger quantities of tissue were required and dog cerebral cortex was used. Craniotomy was performed on dogs under general anesthesia, the brain was removed and cerebral cortical gray and white matter dissected out and weighed; 10% homogenates were prepared and the fractions isolated as described for mouse brain. The nuclear fraction, crude mitochondria and microsomes were obtained from gray matter and myelin from white matter. The protein content was determined and

suspensions were then diluted with 0·32 M sucrose solution to give the required protein concentrations. These fractions were incubated with DPH-^{14}C for 15 min in a shaking water bath at 37°. Human plasma obtained from the blood bank was used to evaluate plasma binding.

Binding studies. Several methods for measuring drug binding to homogenates and subcellular fractions were evaluated and the ultrafiltration method[12,16] was found to be the most consistent and reproducible. One-ml samples of homogenates or subfraction suspensions were placed in dialysis tubing which was knotted at both ends. The dialysis bag was suspended in a 50-ml polyethylene tube with the upper end held in place by the cap of the tube and the lower end placed just above the bottom of the tube. The tubes were centrifuged for 10 min at 800 *g* and 0° to remove water from the dialysis tubing.[17] The tubing and centrifuge tube were carefully wiped dry and then recentrifuged at 1100 *g* for 60 min. A small volume (15–100 μl) of protein-free fluid was thus obtained which was removed for radioactivity determination. Binding was calculated by the formula:

$$\% \text{ binding} = \frac{\text{cpm}_{in} - \text{cpm}_{out}}{\text{cpm}_{in}} \times 100$$

where cpm_{in} is counts/min/ml inside the dialysis tubing before centrifugation and cpm_{out} is the counts/min/ml in the ultrafiltrate.

Per cent binding was found to be independent of DPH concentration between 10^{-5} M and 10^{-8} M, and a concentration of approximately 10^{-6} M was used in these studies. Binding of DPH to the cellulose tubing accounted for a small percentage of the total radioactivity over a wide range of values and no correction for this binding was made.

Some fractions were evaluated for binding after sonication. In these experiments a Biosonik sonicator was used at maximum probe intensity for 30 sec.

Protein and radioactivity determination. Samples (0·1 ml) removed for radioactivity determination were placed directly into scintillation vials and solubilized in 0·3 ml Soluene (Packard Instruments). Bray's solution[18] was added as a scintillation medium and a clear solution without particulate matter obtained. Samples were prepared in duplicate and counted in a Nuclear-Chicago liquid scintillation counter with a counting error of less than 3 per cent. Protein was determined in duplicate by the method of Lowry *et al.*[19]

RESULTS

In vivo. The distribution of radioactivity among the various fractions derived from mouse brain is shown in Table 1. At 2 hr, approximately 26 per cent of the total radioactivity is associated with particulate fractions, the remainder with the supernatant. The bulk of the bound radioactivity is associated with the crude mitochondrial fraction, with only 3 per cent of total radioactivity in the nuclear fraction and less than 1 per cent in the microsomal fraction. In animals sacrificed at 14 hr, a similar distribution of radioactivity associated with the crude mitochondrial fraction was observed. Nuclear and microsomal binding was not different from that of the 2-hr group.

Incubation of brain homogenates *in vitro* with DPH-^{14}C followed by differential centrifugation produced a pattern of distribution similar to that observed in the experiments *in vivo*, although there was a generalized decrease in particulate binding.

TABLE 1. SUBCELLULAR DISTRIBUTION OF DPH-^{14}C IN MOUSE BRAIN

Tissue fraction	2 hr		14 hr		*In vitro*	
	(%)*	RSA†	(%)*	RSA†	(%)*	RSA†
Homogenate	100	1·00	100	1·00	100	1·00
Nuclei-fragments	3	0·33 (0·04)	2	0·26 (0·02)	1	0·18 (0·04)
Crude mitochondria	22	0·63 (0·04)	24	0·64 (0·06)	17	0·50 (0·01)
Purified mitochondria		0·04 (0·02)		0·03 (0·01)		0·03 (0·01)
Synaptosomes		0·11 (0·03)		0·09 (0·02)		0·10 (0·02)
Myelin		1·10 (0·06)		1·13 (0·05)		1·68 (0·13)
Microsomes	1	0·14 (0·03)	1	0·12 (0·03)	1	0·08 (0·01)
Supernatant	74	1·91 (0·08)	73	1·97 (0·13)	81	2·18 (0·15)

* Per cent of recovered radioactivity; actual recovery: 96–102 per cent.

† RSA = relative specific activity = (cpm/mg protein in fraction)/(cpm/mg protein in whole homogenate). Homogenate radioactivity was 7233 cpm/mg protein, 5192 cpm/mg protein and 31,327 cpm/mg protein for 2 hr, 14 hr and *in vitro* respectively. Each value represents the mean of four to six experiments performed in duplicate (± S.E.).

The relative radioactivity within each group of experimental conditions was calculated by comparing the activity of each fraction to the activity in the total homogenate (Table 1). When expressed in these terms, it can be seen that there is no difference between the animals sacrificed at 2 and 14 hr after injection. The data *in vitro* show the same general pattern of distribution.

Fractionation of the crude mitochondrial fraction suggests that the myelin fraction is associated with the highest levels of radioactivity, and the purified mitochondria and synaptosomes are associated with little radioactivity.

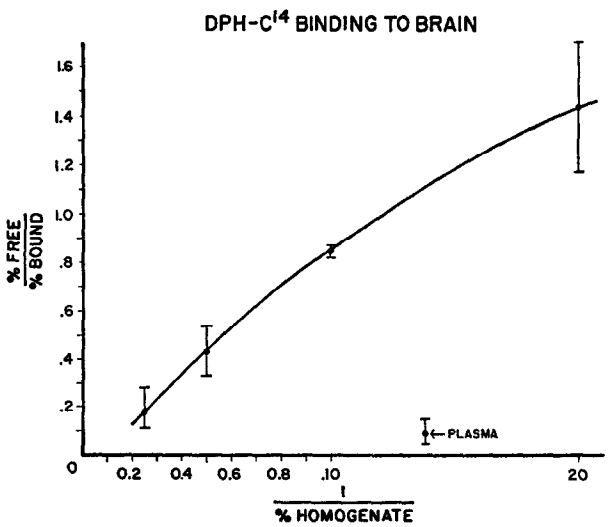

FIG. 1. Binding of DPH-^{14}C to mouse brain homogenates. Homogenates of varying concentration (w/v) were incubated with DPH-^{14}C and binding was measured by ultrafiltration. Binding to human plasma was measured by the same technique and is shown on the lower right, but is not related to the horizontal coordinate. Each point is the mean (±range) of three experiments performed in duplicate.

To assess further the nature of this binding, a sample of the whole homogenate and each of the subcellular fractions were suspended in 11 ml of 0·32 M sucrose and centrifuged at 100,000 *g* for 60 min. The pellet thus obtained was again suspended in sucrose solution and the washing procedure repeated. Radioactivity of the whole homogenate was reduced to 1 per cent of the original value and binding to individual fractions was almost negligible.

Ultrafilatration studies. Mouse brain homogenates were preincubated with DPH-^{14}C for 15 min at 37° and subjected to ultrafiltration. Figure 1 shows the relative binding to various concentrations of homogenates plotted as reciprocals.[12] In 40% homogenates, 85 per cent of the DPH is bound, and in 5% homogenates, 41 per cent is bound. Extrapolation of this curve to a theoretical 100% homogenate indicates that binding would be over 90 per cent. A similar curve is obtained if the data are plotted on the basis of protein content. Human plasma studied by this method bound 92 per cent of DPH. Homogenates subjected to sonication for 30 sec showed similar binding values.

Subcellular fractions isolated from dog brain and then incubated with DPH-^{14}C for 15 min at 37° were also studied by ultrafiltration after adjusting the concentration of protein. Table 2 shows that binding to the various fractions is fairly uniform and

TABLE 2. DPH-^{14}C BINDING TO PRE-ISOLATED FRACTIONS FROM DOG BRAIN*

Fraction	Protein (mg/ml)	Bound (%)
Nuclei fragments		
Whole	14·6	64·9
	7·3	50·3
Sonicated	15·0	66·0
	7·6	52·1
Crude mitochondria		
Whole	15·2	67·7
	7·6	51·9
Sonicated	15·2	68·9
	7·6	56·1
Microsomes	15·8	68·3
	12·8	63·5
	7·8	51·4
Myelin	4·4	43·9
Supernatant	0·93	34·1
	0·47	26·7

* Each value represents the mean of three experiments performed in duplicate.

can be related to total protein content. Nuclei, crude mitochondria and microsomes bind approximately 65 per cent at protein concentrations of 15 mg/ml and 50 per cent at half that concentration of protein. Myelin was not obtained in large enough quantity to compare directly, but appears to bind DPH in a similar way. The supernatant fractions were more dilute but bound 40 per cent of the DPH at a concentration of about 1 mg/ml of protein. Sonication of the crude mitochondria did not appear to affect binding capacity.

DISCUSSION

The degree of drug binding to tissue constituents is defined by the conditions used to measure such binding. Even in a relatively simple system such as serum, factors such as pH, ionization, concentration of drug, and protein can affect apparent binding.[20] Binding measurements in a more complex system such as a homogenate where one is dealing with membrane-bound tissue present even greater difficulty. This is well illustrated in the present investigation where it was found that washing the homogenate by centrifugation at 100,000 *g* for 1 hr almost completely removed the "bound" drug. A similar finding was noted by Nielsen and Cotman,[11] who were able to remove almost all of the bound drug by pelleting homogenates four times at 120,000 *g* for 45 min. With subcellular particles, another degree of complexity is added, as the very methods used to separate the subcellular fractions can remove associated drugs. The microsomal fraction is defined by centrifugation at 100,000 *g* and the method used for separation of microsomes was shown to remove DPH-^{14}C from particulate fractions. For this reason the prior isolation of the subcellular fraction with subsequent incubation with radioactivity was selected as the best method of avoiding this uncertainty. When this method was applied to DPH-^{14}C, we were able to demonstrate a good correlation between binding capacity and tissue protein content irrespective of the subcellular fraction utilized.

The use of low speed centrifugation of tissue suspensions in dialysis tubing has been applied to measure the binding of several compounds. Cho and Curry[16] used this technique to measure binding of an experimental hypotensive agent to salivary gland homogenates, and it has also been applied to the measurement of guanethidine.[12] We initially evaluated this method using whole brain homogenates of various dilutions and were able to demonstrate a relationship between tissue concentration and per cent binding, as is illustrated in Fig. 1. This method was then applied to the measurement of binding of specific subcellular fractions with the assumption that they would behave in the same fashion as whole tissue homogenates. This assumption was tested further by subjecting the fractions to sonication, which presumably disrupted the particulate membranes and produced a more homogeneous suspension of tissue constituents. The sonicated fragments bound DPH-^{14}C to the same degree as did the intact subcellular particles.

These results are in agreement with the studies of Firemark *et al.*[3] These investigators used tissue slices and evaluated binding by applying a force of 30 cm mercury to the tissue and measuring the DPH content of an ultrafiltrate passing through a cellulose membrane at 37°. They reported brain tissue binding of diphenylhydantoin to be approximately 90 per cent. Extrapolation of the curve in Fig. 1 would indicate that binding to a theoretical 100% homogenate would be greater than 90 per cent.

Our findings indicate that diphenylhydantoin is reversibly bound to various subcellular components of brain. This binding capacity appears to correlate with the quantity of tissue as measured by protein content and it is not dependent on maintenance of subcellular structure, since sonicated tissue exhibits the same binding properties. We are unable to confirm Kemp and Woodbury's report[6] of increasing microsomal binding with time, and, in fact, found that the microsomes bind only a small percentage of total radioactivity, which did not change significantly from 2 to 14 hr. Furthermore, the distribution of radioactivity in the homogenate experiments *in vitro* in the present study suggests that this binding is not dependent on time.

Kemp and Woodbury[6] conclude that DPH binding to brain is covalent, largely on the basis of paper chromatographic studies. Neither we nor others have confirmed this finding.[9-11] The ultrafiltration method employed in the present study appears to be a more reliable method for measuring binding to subcellular fractions.

DPH binding to previously separated subcellular fractions differs from binding to fractions separated after injection *in vivo* or incubation *in vitro* prior to separation. In a complex mixture there are methodological factors favoring redistribution and binding to specific subcellular components. For example, the association of DPH-^{14}C with the myelin subfraction appears greater than with any of the other particulate fractions. Other authors using discontinuous gradients noted similar results.[11] However, when other methods of isolating myelin are employed, myelin binds relatively little DPH. Nielsen and Cotman[11] found that lipids extracted from brain bound less DPH than did soybean phospholipids, and in the ultrafiltration study we found binding to pre-isolated myelin could be related to protein content, as was true in other fractions. The apparent affinity to myelin may represent a redistribution phenomenon with some of the drug removed from other fractions during centrifugation at 53,000 *g*, as well as contamination from the supernatant remaining in the upper layer, which also contains myelin. Redistribution and trapping of DPH in membrane-bound structures may also explain the discrepencies among the reports on subcellular binding of DPH.[9]

A number of other variables are known to affect drug binding. These studies were conducted at a pH (6·7) at which 99·9+ per cent of the DPH is in the un-ionized form. At higher pH greater binding might be expected.[3] Concentration of drug was not a factor in this investigation. Wilensky and Lowden[10] have reported that DPH binding is independent of ionic strength and osmolarity.

The relationship between DPH binding to brain and its mechanism of action is unknown. The binding demonstrated in this study appears to be nonspecific, but does not exclude the possibility of binding to a specific protein which was not recognized under these experimental conditions.[21] Finally, it is recognized that the distribution of DPH in intact brain may not correspond to the binding properties of isolated fractions, as techniques *in vitro* by their very nature disrupt normal membrane organization and relationships. Electron microscopic autoradiography would be useful in resolving this uncertainty.

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REFERENCES

1. S. W. Rose, L. D. Smith and J. K. Penry, *Blood Level Determinations of Antiepileptic Drugs*, pp. 1–18. National Institute of Neurological Diseases and Stroke, Bethesda, Md. (1971).
2. H. M. Triedman, R. A. Fishman and M. D. Yahr, *Trans. Am. neurol. Ass.* 166 (1960).
3. H. Firemark, C. F. Barlow and L. J. Roth, *Int. J. Neuropharmac.* **2**, 25 (1963).
4. E. L. Noach, D. M. Woodbury and L. S. Goodman, *J. Pharmac. exp. Ther.* **122**, 301 (1958).
5. J. W. Kemp and D. M. Woodbury, *Pharmacologist* **4**, 159 (1962).
6. J. W. Kemp and D. M. Woodbury, *J. Pharmac. exp. Ther.* **177**, 342 (1971).
7. D. M. Woodbury, *Epilepsia* **10**, 121 (1969).
8. M. A. Goldberg and T. Todoroff, *Neurology, Minneap.* **22**, 410 (1972).
9. T. Yanagihara and A. Hamberger, *J. Pharmac. exp. Ther.* **179**, 611 (1971).
10. A. J. Wilensky and J. A. Lowden, *Can. J. Physiol Pharmac.* **50**, 346 (1972).
11. T. Nielsen and C. Cotman, *Eur. J. Pharmac.* **14**, 344 (1971).

12. L. S. SCHANKER and A. S. MORRISON, *Int. J. Neoropharmac.* **4,** 27 (1965).
13. V. P. WHITTAKER, *Biochem. J.* **72,** 694 (1959).
14. K. M. TAYLOR and S. H. SNYDER, *J. Neurochem.* **19,** 341 (1972).
15. S. TUCEK, *J. Neurochem.* **13,** 1317 (1966).
16. A. K. CHO and S. H. CURRY, *Biochem. Pharmac.* **18,** 511 (1969).
17. D. BORGA, D. L. AZARNOFF, G. P. FORSHELL and F. SJOQVIST, *Biochem. Pharmac.* **18,** 2135 (1969).
18. G. A. BRAY, *Analyt. Biochem.* **1,** 279 (1960).
19. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
20. A. GOLDSTEIN, L. ARONOT and S. KALMAN, in *Principles of Drug Action,* p. 136. Harper & Row, New York (1969).
21. J. T. FARROW and H. VANVUNAKIS, *Biochem. Pharmac.* **22,** 1103 (1973).