AGE-DEPENDENT CHANGES IN PHENYTOIN TISSUE BINDINGS IN RATS: COMPARISON BETWEEN IN VIVO AND IN VITRO TISSUE-TO-BLOOD PARTITION COEFFICIENTS ($K_p$ VALUES) OF PHENYTOIN

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Age-dependent changes in phenytoin tissue bindings in rats were investigated by equilibrium dialysis using serum and 10% tissue (brain, lung, liver, kidney and muscle) homogenates. All percentages of phenytoin bound to serum and tissue homogenates were independent of the initial phenytoin concentration (2 to 25 µg/ml) in 1-d, 1-, 3- and 8-week-old rats. The percentages bound to serum, brain, liver, kidney and muscle in newborn rats (1-d-old rats) were lower than those in 8-week-old rats and the percentages bound increased gradually in the growth process. However, those in lungs were constant in all ages of rats. It was assumed that the age-dependent changes in phenytoin tissue binding were caused by the changes in the quantities of tissue constituents to which phenytoin bound in the growth process. Tissue-to-blood partition coefficients ($K_p$ values) were calculated from in vitro tissue binding data and the pH-difference across the cell membrane. These $K_p$ values were in good agreement with the in vivo $K_p$ values reported previously. It was concluded that the age-dependent changes in phenytoin tissue distribution were caused by the age-dependent changes in phenytoin binding to blood constituents and tissues but that the change of phenytoin blood binding contributed to the age-dependent changes in $K_p$ values of phenytoin more than to phenytoin tissue binding and consequently the $K_p$ values of phenytoin decreased as rats grew.

**Keywords** — phenytoin; tissue binding; tissue-to-blood partition coefficient; age-dependent change; equilibrium dialysis

INTRODUCTION

Phenytoin, an acidic drug which binds relatively highly to serum protein and tissue constituents, is a widely used anticonvulsant agent and is frequently administrated to newborn infants in clinical drug therapies.

In a previous paper,¹ we reported the age-dependent changes of phenytoin distribution in rats. That is to say, the distribution volume of phenytoin decreased in the growth process of rats. Blood free percentages of phenytoin in 1-d and 1-week-old rats were larger than that in 8-week-old rats. However, the correlation between blood free percentages and distribution volumes of phenytoin ($p > 0.2$) in the growth process was not statistically significant. This suggests that, for phenytoin, not only the changes of blood free fractions but also other factors affect the changes of the distribution volumes.

Drug binding to tissue constituents greatly affects the distribution volume and the studies of drug bindings to tissues are often performed using diluted tissue homogenates for methodological reasons. Lin et al.² reported that in vivo tissue distribution of ethoxybenzamid could be estimated from in vitro tissue binding data using tissue homogenates. Harashima et al.³ suggested that the extensive tissue distribution of quinidine observed in vivo might be explained by tissue binding and the pH-difference across the cell membrane in most tissues.

On the other hand, Igari et al.⁴ failed to predict tissue-to-blood distribution ratios of hexobarbital, phenobarbital and thiopental from tissue binding and Mintum et al.⁵ reported that tissue distribution of tetraethylammonium ion was not explained by its binding to tissue homogenates but could be explained by in vitro tissue uptake data. Thus, it is necessary to clarify whether tissue distribution of particular drugs can be estimated from in vitro tissue binding data using tissue homogenates.

In this paper, we studied the age-dependent changes in phenytoin tissue bindings in rats and
estimated the tissue distribution of phenytoin in variously aged rats. The in vitro tissue binding data were obtained by using tissue homogenates in order to determine whether the age-dependent changes in phenytoin tissue distribution were caused by changes in phenytoin binding to both blood constituents and tissues.

**MATERIALS AND METHODS**

**Chemicals** — 5,5-[4-14C]-diphenylhydantoin (14C-phenytoin) and non-radioactive phenytoin were purchased from New England Nuclear, Boston, MA, U.S.A. and Aldrich Chemical Company, Milwaukee, WI, U.S.A., respectively. Phenytoin was solubilized with a minimum amount of dilute sodium hydroxide and diluted with non-radioactive phenytoin to 10.4 μCi/5 mg/ml in normal saline. All other chemicals were of analytical grade and used without further purification.

**Animals** — Pregnant Wistar rats and 8-week-old male Wistar rats were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. One day, 1- and 3-week-old male rats were obtained after the delivery of the pregnant rats in our laboratory.

**Tissue Preparation** — Blood samples were obtained from rats to which no phenytoin or anticoagulants had been administered and were centrifuged at 3000 rpm for 10 min at 4°C to obtain serum samples. The one, 3- or 8-week-old rats were cannulated in the portal vein under ether anesthesia. After washing blood from the lumbar vein with ice-cold normal saline being pumped into the portal vein, brains, lungs, livers, kidneys and femoral muscles were excised, pooled and frozen at -20°C until study. Because it was very difficult to cannulate 1-d-old rats in the portal vein, the rats were decapitated and each tissue was excised, rinsed with ice-cold normal saline, blotted dry and frozen at -20°C until study. Ten % w/v tissue homogenates were prepared in 0.01 M phosphate buffer containing 0.15 M KCl (pH 7.0) on ice using a glass homogenizer.

**Phenytoin Binding to Serum and Tissue Homogenates** — Phenytoin binding to serum and tissue homogenates was determined by equilibration dialysis. For the binding studies, a dialysis cell or semimicrodialysis cell having 2 chambers separated by a cellophane membrane for dialysis (Cellophane Tubing-Seamless, Union Carbide Co., U.S.A.), presoaked in 0.01 M phosphate buffer containing 0.15 M KCl (either pH 7.0 or 7.4) was used. The non-diluted serum or the tissue homogenates was spiked with 14C-phenytoin solution at initial concentrations ranging from 2 to 25 μg/ml. The serum (200 μl) or tissue homogenates (1 ml) were added to one side of the membrane and the same volume of phosphate buffer containing 0.15 M KCl, pH 7.4 for serum or pH 7.0 for tissue homogenates was added to the other side. After shaking the dialysis cells at 4°C for 30 h, a sample of the serum, tissue homogenate or buffer was taken and dissolved in 1 ml of Soluene-350 (Packard Instrument Co., Dowers Grove, U.S.A.) and then 10 ml of the scintillation fluid [2,5-diphenyl-oxazole, 5 g; 1,4-bis-2-(5-phenyloxazolyl)-benzene, 0.3 g; toluene, 700 ml; Triton X-100, 300 ml] were added. These samples were allowed to stand for 1 d in darkness and then radioactivities were determined with an Aloka LSC-903 liquid scintillation system (Aloka Co., Ltd., Tokyo, Japan).

**Determination of Tissue Water Content** — About 200 mg of tissues were weighed precisely and heated at 100°C to constant weight. From the differences of tissue weights before and after heating, the water content of tissues was calculated.

**Calculation of Phenytoin Bindings to Tissues from Those to Tissue Homogenates** — The binding of a drug is generally described by a Langmuir-type equation:

\[ C_b = n \cdot p \cdot k \cdot C_t/(1 + k \cdot C_t) \]  

(1)

where \( C_b \), \( C_t \), \( p \), \( k \) and \( n \) are bound drug concentration, free drug concentration, concentration of binder, the association constant and the number of binding sites, respectively. When \( k \cdot C_t \) is much smaller than 1, Eq. 1 becomes Eq. 2 which shows that the fraction bound is independent of the drug concentration. 

\[ C_b/C_t = n \cdot k \cdot p \]  

(2)

The fraction bound (\( R \)) to the tissue homogenate is defined by:

\[ R = C_b/(C_b + C_t) \]  

(3)

From Eqs. 2 and 3, \( n \cdot k \) is calculated by:

\[ n \cdot k = R/(p \cdot R - p \cdot R) \]  

(4)

If the drug binding characteristics (\( n \cdot k \)) are not changed by the concentration of tissue homogenates and the fraction bound to tissue homogenates (of which concentration of the binder is \( p' \)) is \( R' \), the next equation is given as follows:

\[ R'/(1 - R') = R \cdot p'/(p - p \cdot R) \]  

(5)
Rearrangement of Eq. 5 yields:

$$R' = R \cdot p'/(p - p \cdot R + p' \cdot R)$$  \hspace{1cm} (6)

When the fraction bound to the 10% w/v tissue homogenate is $R$, the fraction bound to tissue (not homogenized, then $p' = 10 \cdot p$), $R'$ is calculated by:

$$R' = 10 \cdot R/(1 + 9 \cdot R)$$  \hspace{1cm} (7)

Calculation of **In Vitro Tissue-to-Blood Partition Coefficients ($K_p$ Values)** — Considering the phenytoin binding to blood and tissue and the pH-difference across the cell membrane that will cause a difference in unbound concentration between blood and tissues, the $K_p$ value can be expressed by:

$$K_p = q \cdot f_B/f_T$$  \hspace{1cm} (8)

$$q = (1 + 10^{pH_i - pK_a})/(1 + 10^{pH_e - pK_a})$$

$$f_B = f_S/RB$$

$$f_T = 1 - R'$$

where $pH_i$, $pH_e$, $pK_a$, $f_B$, $f_T$, $f_S$ and $RB$ are the pH of tissue, pH of serum, $pK_a$ of phenytoin, blood free fraction, tissue free fraction, serum free fraction and blood-to-plasma partition coefficient of phenytoin, respectively. The values of 7.0 for $pH_i$, 7.4 for $pH_e$, 8.06 for $pK_a$ were used for the calculation. The $RB$ for phenytoin in 1-d, 1-, 3- and 8-week-old rats were 1.36, 1.01, 0.941 and 1.02, respectively. The value for $f_S$ was obtained experimentally by equilibrium dialysis. $R'$ was calculated by Eq. 7 using the data on the phenytoin binding to 10% w/v tissue homogenates.

**RESULTS**

**Phenytoin Binding Characteristics to Serum and Tissue Homogenates**

Figure 1 (A) shows the phenytoin bindings to the undiluted serum and 10% tissue (brain, lung, liver, kidney and muscle) homogenates in 8-week-old rats at various phenytoin concentrations. The percentages of phenytoin bound to serum and tissue homogenates were all independent of the initial phenytoin concentrations and were constant. We also examined the phenytoin binding to the serum and tissue homogenates obtained from 1-d, 1- or 3-week-old rats at the same initial concentration. The data obtained suggested that, in rats of any age the binding percentages to serum and to any tissue homogenates were concentration-independent.

Figure 1 (B) shows the mean binding percentages to serum and 10% tissue homogenates in variously aged rats. The binding percentages to serum, brain, liver, kidney and muscle in 1-d-old rats were lower than those in 8-week-old rats and the binding percentages increased gradually in the growth process. On the other hand, the binding percentages in lungs were constant in rats of any age.

The effect of concentration of various tissue homogenates on phenytoin binding was examined in 8-week-old rats with 5, 10, 20 and 30% w/v tissue homogenates at the initial phenytoin concentration of 10 µg/ml. As shown in Fig. 2, phenytoin bindings were directly proportional to the concentration of tissue homogenates. This shows that the binding characteristics ($n \cdot k$, this corresponds to the slope in Fig. 2) of phenytoin were not changed by the concentration of tissue homogenates.

**In Vitro Tissue-to-Blood Partition Coefficients**

Table I summarizes the phenytoin bindings to tissues calculated by Eq. 7 in variously aged rats. Phenytoin bindings were considerably high in all tissues studied. Table II lists the $K_p$ values calculated by Eqs. 7 and 8 using *in vitro* tissue binding data and the *in vivo* $K_p$ values are the data reported previously.

**DISCUSSION**

Because of methodological difficulties, the studies of drug binding to tissues are often performed using the diluted tissue homogenates. In this experiment, we studied the phenytoin tissue binding by equilibrium dialysis using 10% tissue homogenates.

It was reported that the fraction of drug bound to proteins tends to decrease with an increase in temperature for a number of drugs and Lunde et al. reported that the free fraction of phenytoin was greater at 37 °C than at room temperature. However, Pantarotto et al. reported that phenytoin was rapidly metabolized during incubation at 37 °C in rat liver microsomal preparations. Therefore, we performed binding studies at 4 °C to prevent both drug metabolism in the tissue homogenates and degradation of tissues during equilibrium dialysis.

Ludden et al. found that phenytoin binding to lung and liver pieces in rats was independent of phenytoin concentration over at least 1000-fold range. In this experiment, using serum and homogenates of brain, lung, liver, kidney and muscle, phenytoin binding was also independent of phenytoin concentration and
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was constant (Fig. 1). Thus, these results showed that Eq. 2 adequately describes the independence of phenytoin binding to serum and tissue homogenates. The percentages bound to serum, brain, liver, kidney and muscle in infant rats were lower than those in 8-week-old rats and increased gradually in the growth process. Fichl et al.\textsuperscript{13} reported that phenytoin binding to muscle increased after lipid removal and pointed out that phenytoin binding to muscle could not be explained solely in terms of binding to lipids. Goldberg and Todoroff found that phenytoin bindings to brain, liver, heart and kidney occurred to the same extent based on protein content. Moreover, they reported that phenytoin might bind nonspecifically to many tissue proteins, irrespective of their origin,\textsuperscript{14} and that phenytoin also bound phospholipids in brain.\textsuperscript{15}

**FIG. 1.** Phenytoin Bindings to Serum and Tissue Homogenates

(A) Phenytoin bindings to serum and 10% tissue homogenates in 8-week-old rats at various phenytoin concentrations. (B) The mean phenytoin binding percentages to serum and 10% tissue homogenates in variously aged rats. Each point represents the mean ± S.D. of 3 cases. The points without vertical bars have smaller S.D. values than the symbols. a) Significantly different from the value of 8-week-old rats at $p < 0.01$. b) Significantly different from the value of 8-week-old rats at $p < 0.05$.\textsuperscript{15}
FIG. 2. Effect of Concentration of Tissue Homogenate on Phenytoin Binding
Each point represents the mean ± S.D. of 3 cases. The point without vertical bars have smaller S.D. values than the symbols.

TABLE I. Percentages Bound of Phenytoin to Serum and Tissues in Variously Aged Rats

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Brain</th>
<th>Lung</th>
<th>Liver</th>
<th>Kidney</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-d-old</td>
<td>79.55±2.51</td>
<td>85.95±0.44</td>
<td>91.01±1.16</td>
<td>94.85±0.88</td>
<td>89.75±0.42</td>
<td>86.15±0.31</td>
</tr>
<tr>
<td>1-week-old</td>
<td>84.52±1.20</td>
<td>89.39±0.26</td>
<td>90.52±0.23</td>
<td>96.39±0.08</td>
<td>91.15±0.30</td>
<td>87.09±0.89</td>
</tr>
<tr>
<td>3-week-old</td>
<td>91.81±0.51</td>
<td>91.03±0.17</td>
<td>89.39±0.81</td>
<td>96.35±0.22</td>
<td>92.66±0.79</td>
<td>91.36±0.22</td>
</tr>
<tr>
<td>8-week-old</td>
<td>94.33±0.21</td>
<td>91.01±0.32</td>
<td>90.39±0.28</td>
<td>96.38±0.19</td>
<td>94.01±0.21</td>
<td>90.93±0.60</td>
</tr>
</tbody>
</table>

Data are calculated by Eq. 7 (except for serum). Each percentage shown is the mean ± S.D. of 3 cases. The initial phenytoin concentration range used in this study was from 2 to 25 μg/ml.

TABLE II. In Vitro and in Vivo Tissue-to-Blood Partition Coefficients of Phenytoin in Variously Aged Rats

<table>
<thead>
<tr>
<th></th>
<th>1-d-old</th>
<th>1-week-old</th>
<th>3-week-old</th>
<th>8-week-old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vitro</td>
<td>In vivo</td>
<td>In vitro</td>
<td>In vivo</td>
</tr>
<tr>
<td>Brain</td>
<td>0.956</td>
<td>1.19</td>
<td>1.29</td>
<td>1.18</td>
</tr>
<tr>
<td>±0.121</td>
<td>±0.105</td>
<td>±0.081</td>
<td>±0.057</td>
<td>±0.148</td>
</tr>
<tr>
<td>Lung</td>
<td>1.49</td>
<td>2.11</td>
<td>1.45</td>
<td>1.44</td>
</tr>
<tr>
<td>±0.266</td>
<td>±0.436</td>
<td>±0.170</td>
<td>±0.073</td>
<td>±0.213</td>
</tr>
<tr>
<td>Liver</td>
<td>2.61</td>
<td>2.85</td>
<td>3.81</td>
<td>3.11</td>
</tr>
<tr>
<td>±0.549</td>
<td>±0.115</td>
<td>±0.052</td>
<td>±0.187</td>
<td>±0.486</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.31</td>
<td>1.51</td>
<td>1.55</td>
<td>1.53</td>
</tr>
<tr>
<td>±0.170</td>
<td>±0.273</td>
<td>±0.197</td>
<td>±0.131</td>
<td>±0.293</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.969</td>
<td>1.02</td>
<td>1.06</td>
<td>1.16</td>
</tr>
<tr>
<td>±0.121</td>
<td>±0.015</td>
<td>±0.075</td>
<td>±0.006</td>
<td>±0.190</td>
</tr>
</tbody>
</table>

Each partition coefficient shown is the mean ± S.D. of 3 separate experiments. The data of in vivo are reported previously. 11
FIG. 3. Changes in Water Content in Variously Aged Rats
Each point represents the mean ± S.D. of 3 cases. The points without vertical bars have smaller S.D. values than the symbols. a) Significantly different from the value of 8-week-old rats at p < 0.01. b) Significantly different from the value of 8-week-old rats at p < 0.05.

Wilensky and Lowden reported that the affinity of phenytoin was greatest for fractions rich in plasma membranes in brain, liver and kidney.\(^{16}\) In the above reports, the constituents in tissues to which phenytoin binds were not clarified. On the other hand, it is well known that in newborn infants the total body water in each tissue as a percentage of body weight is very high.\(^ {17}\) Figure 3 shows the changes in water content in tissues as rats grew. In all tissues except lung, water content was large in infant rats and gradually decreased in the growth process. These results suggest that the quantities of tissue constituents to which phenytoin binds change in the growth process and that this may cause the changes in phenytoin bindings to tissues in the growth process.

For the extrapolated calculation of drug bindings to tissues from the drug bindings to tissue homogenates, we assumed that the binding characteristics \((n \cdot k)\) of the drug were not changed by the concentration of tissue homogenates and accordingly, Eq. 7 was derived. This assumption might be reasonable for phenytoin because the value of \(C_b/C_t\) were linear to 30% concentration of all tissue homogenates studied (Fig. 2). The use of the diluted tissue homogenates is mainly due to the experimental difficulty in preparing higher concentrations of tissue homogenates and performing equilibrium dialysis experiments with such homogenates. Phenytoin bindings to all tissues studied were considerably high (over

FIG. 4. The Relationship between the \(K_p\) Values Obtained from in Vivo Intravenous Bolus Injection and in Vitro Tissue Binding in Variously Aged Rats
Each point represents the mean ± S.D. of 3 separate experiments. The points without vertical or horizontal bars have smaller S.D. values than the symbols. The data of in vivo intravenous bolus injection are reported previously.\(^ {13}\) A solid line represents a 1:1 relationship. Key: ▲, brain; ○, lung; O, liver; □, kidney; △, muscle.
85% bound). This result was similar to that reported by Ludden et al.\textsuperscript{12)} (in the case of lung and liver) and Goldberg et al.\textsuperscript{18)} (in the case of brain) and this may be a reason that phenytoin showed the relatively large distribution volume (about 1 l/kg\textsuperscript{11)} in spite of the relatively high blood binding.

Figure 4 shows good agreement of the relationship between the $K_p$ values obtained from in vivo intravenous bolus injection and in vitro tissue binding in variously aged rats. This result shows that tissue distribution of phenytoin can be explained by extensive tissue and blood binding and that the age-dependent changes in
Phenytoin tissue distribution are caused by the age-dependent changes in phenytoin binding to blood constituents and tissues.

Tissue-to-blood unbound concentration ratio ($K_{p,f}$ value), the parameter that is excluded the influence of fluctuation in blood free fraction from $K_p$ value, is defined by 19):

$$K_{p,f} = \frac{K_p}{f_B}$$

Figure 5 shows the relationship between the age of rats and both in vivo and in vitro $K_{p,f}$ value (A) or $K_p$ value (B) in various tissues. In most tissues, $K_{p,f}$ values increased as rats grew. On the other hand, $K_p$ values decreased. These results show that the change of phenytoin blood binding contributed to the age-dependent changes in $K_p$ values of phenytoin more than to phenytoin tissue binding and consequently $K_p$ values of phenytoin decreased as rats grew.

REFERENCES