In the second, the binding constant is two orders of magnitude smaller; the process is much less exothermic and the entropy is much less negative. This suggests that, although an ionic interaction is maintained, in the second type of binding site there is also an important hydrophobic contribution which is much greater than in the first type of site.

In view of the variation in the quantum yield of fluorescence of salicylate upon binding to HSA, the presence of another substance that might compete with the salicylate for the same binding sites on the protein should give rise to a decrease in the intensity of emission. Measurements of the fluorescence of salicylate ($5{\cdot}0 \times 10^{-6}$ M) in the presence of HSA ($5{\cdot}0 \times 10^{-5}$ M) were performed with different concentrations of imidazole corresponding to molar imidazole/salicylate ratios of 5, 2, 1 and 0·5. In no case were any differences detectable in the intensity of fluorescence on comparing solutions with the same concentration of salicylate in the absence and presence of imidazole. It should be noted that there is no interference due to absorbance of imidazole at an excitation wavelength of 310 nm. This finding is in agreement with the observation, found using ultrafiltration measurements, that imidazole does not compete with salicylate for the same binding sites on HSA (Rodrigo et al 1988).

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Enhancement of phenytoin binding to tissues in rats by heat treatment

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Abstract—Phenytoin binding to heat-treated tissue homogenates has been examined to characterize the phenytoin binding to tissues. The binding to the heat-treated tissue homogenates was enhanced in all tissues studied compared with controls. The heating might produce the changes in conformation of proteins in tissues and then enhance phenytoin binding to tissue homogenates.

Phenytoin is a widely used anticonvulsant agent in clinical drug therapies and we succeeded in estimating in-vivo tissue-to-blood partition coefficients of phenytoin in variously aged rats from in-vitro binding data using serum and tissue homogenates (Kato et al 1987). However, even in brain which is a target organ for phenytoin, the factors governing the tissue distribution of phenytoin are not clarified and conflicting data have been reported regarding the binding characteristics of phenytoin to brain membranes. Burnham et al (1981) reported that phenytoin binds in a saturable and reversible manner to at least two sites in brain membrane fractions and after heat pretreatment, binding was greatly enhanced, but the saturable binding could no longer be seen. These data agree well with the results of Shah et al (1981) who pointed out, however, that heat treatment enhanced the specific binding of phenytoin to brain membranes. On the other hand, Geary et al (1987) and Goldberg & Todoroff (1976) reported that there are no specific binding sites for phenytoin in brain. Furthermore, Geary et al (1987) pointed out that the treatment of brain section with both formalin and heat enhanced the nonspecific binding compared with the control.

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It is well recognized that drug binding to blood or tissue constituents greatly affects the disposition of the drug. Recently, drug binding to the tissue constituents has stressed as an important pharmacokinetic determinant (Terasaki et al 1984; Harashima et al 1984). Whether this phenomenon found in heat-treated brain is specific to brain, led us to make a preliminary experiment to characterize the phenytoin binding to tissues, so phenytoin binding to heat-treated tissue homogenates was examined.

Materials and methods

Adult male Wistar rats (Shizuoka Laboratory Animal Center, Japan), 270–300 g, were cannulated in the portal vein under ether anesthesia. After washing blood from the lumbar vein by pumping ice-cold saline (0·9%) into the cannulae to exsanguinate tissues, the tissues (brain, lung, liver, kidneys and muscle) were excised, blotted dry and frozen at −20°C until study. 10% w/v tissue homogenates were prepared in 0·01 M phosphate buffer containing 0·15 M KCl (pH 7·0) on ice. Part of the tissue homogenates were bubbled mildly with nitrogen gas to avoid denaturation (oxidation) in phospholipids during heating and then the vessels were sealed with Saran Wrap (Asahi Kasei Kogyo Co., Ltd, Japan). Heat-treated tissue homogenates were prepared by placing them in boiling water for 10 min. [^{14}C]phenytoin (New England Nuclear, USA) was diluted with non-radioactive compound (Aldrich Chemical Company, USA) and spiked into the homogenates at the initial concentration of 10 μg mL^{-1} (20·4 μCi L^{-1}). Phenytoin binding to tissue homogenates was determined by equilibrium dialysis as described previously

(Kato et al 1987). Phenytoin in tissue homogenates and dialysates was assayed using a liquid-scintillation counter (Aloka LSC-903, Japan) with 10 mL of scintillation counting solution (5 g of 2,5-diphenyloxazole and 0·3 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene in 700 mL of toluene and 300 mL of Triton X-100) after tissue homogenates or dialysates had been dissolved in 1 mL of Soluene-350 (Packard Instrument Co., U.S.A.). Student's *t*-test was used for statistical analysis.

Results and discussion

The factors governing the tissue distribution of phenytoin are not clarified yet even in brain which is a target organ for phenytoin. It was reported that phenytoin binds to both protein and phospholipid in tissues (Goldberg & Todoroff 1973, 1976). On the one hand, Goldberg & Todoroff (1976) or Fichtl et al (1980) reported that the phenytoin binding to brain or muscle was enhanced by lipid extraction. They pointed out two possibilities: (i) the removal of lipid unmasked further potential phenytoin binding sites that were associated with lipid in native state; (ii) the organic solvent used for the lipid extraction produced some denaturation in protein with conformational changes that enhanced the binding.

We then attempted to examine phenytoin binding to tissue binding to tissue homogenates in which protein, not phospholipid, was denatured by heating (Harashima et al 1984). The percentages of phenytoin bound to tissue homogenates were independent of the initial phenytoin concentration (2–25 μg mL^{-1}) and were constant (Kato et al 1987) so that in this experiment we selected the initial phenytoin concentration to 10 μg mL^{-1}. Fig. 1 shows phenytoin binding to the heat-treated and control homogenates.

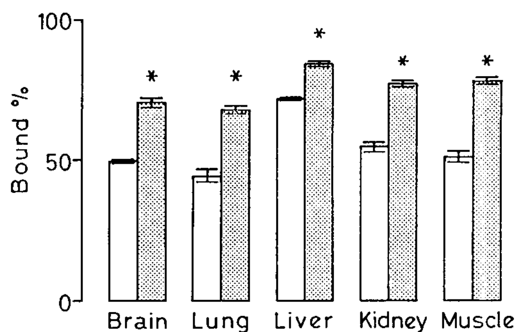

FIG. 1. Effect of heat treatment on phenytoin binding to tissue homogenates. Phenytoin binding to normal (open columns) and heat-treated (dotted columns) 10% w/v tissue homogenates was determined by equilibrium dialysis. The bars give the s.d. (n = 3). * Significantly different from the control value ($P < 0.01$).

In brain, phenytoin binding to the heat-treated homogenate was enhanced 43·8% compared with the control. This agreed well with the results of several investigators (Burnham et al 1981; Shah et al 1981; Geary et al 1987). Burnham et al (1981) and Shah et al (1981) discussed this phenomenon in correlation with the specific binding of phenytoin to brain. However, as shown in Fig. 1, phenytoin binding to the heat-treated tissue homogenates was enhanced considerably not only in brain but also in all tissues studied. This finding suggested that the heating produced the changes in conformation of tissue proteins which resulted in the enhancement of phenytoin binding to the tissue homogenates. The clinical implication of this finding is unknown now but it is unusual and interesting that the denaturalization of protein leads to the enhancement of its activity (for example binding activity or enzyme activity). This finding may be of use in the study of the mechanisms of tissue binding of phenytoin.

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