Determinants of *in Vitro* Binding of Barbiturates by Rat Hepatic Subcellular Fractions* (33006)

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Pentobarbital is a clinically important drug whose pharmacologic effect is terminated largely through biotransformation via the mixed-function oxidase system in the endoplasmic reticulum of hepatic and renal cells (1–3).

In contrast, phenobarbital slowly undergoes metabolism to a single *p*-hydroxy derivative and is also excreted unchanged in the urine (4). The diethyl analogue, barbital, is virtually nonmetabolized; only about 4% is recoverable at 24 hours as metabolites in the rat (5).

The rate of barbiturate metabolism is dependent upon species, age, sex, nutrition, and previous exposure to drugs (6). A mechanism to explain these differences might lie in the ability of the endoplasmic reticulum to localize the substrate. Support is given to this postulate by the recent identification of cytochrome P-450 (7,8), a non-heme ferroprotein component of the reticulum, as a binding site for drugs undergoing oxidation (9).

We have studied the binding of barbiturates by hepatic cell fractions with emphasis on microsomal binding of pentobarbital. It is postulated that binding may be involved in the differences in rate of metabolism among barbituric acid derivatives and in the increase seen with age.

Materials and Methods. Chemicals. Pentobarbital-2-^{14}C (5.97 mC/mmole) and phenobarbital-2-^{14}C (2.22 mC/mmole) was obtained from New England Nuclear Corporation, Boston, Massachusetts, and barbital-2-^{14}C (1.1 mC/mmole) from Tracerlab, Waltham, Massachusetts.

Animals. Rats of the Sprague-Dawley strain were obtained while pregnant from Hormone Assay Laboratories, Chicago, Illinois. After birth, litter size was adjusted to eight.

Preparation of subcellular fractions. Nonfasted animals were sacrificed by decapitation after stunning. The liver was quickly excised, immersed in iced 0.25 *M* sucrose solution, blotted dry, and weighed. It was then perfused until well blanched, and pressed dry until the weight equalled the initial value. This procedure was not possible with the livers from newborn rats.

Subcellular fractions were prepared by the methods of Hogeboom (10) and Munro *et al.* (11) and microsomal subfractions as described by Hallinan and Munro (12). The particulate fractions were resuspended in 0.25 *M* sucrose containing 0.002 *M* $CaCl_2$ and 0.001 *M* $MgCl_2$ (pH 5.2) and diluted to a protein concentration of 0.5–2.0 mg/ml. The final pH was 5.7–6.3.

Incubation studies. Labeled drugs (5×10^{-8} moles in 0.1 ml) were added to 4.9 ml of the subcellular fraction suspension. After mixing, the tubes were incubated for 15 min at 37°C. In displacement studies, 5×10^{-5} moles of unlabeled drug was added and incubation was continued for an additional 15 min. Two ml of cold 10% trichloroacetic acid (TCA) was added and the tubes were centrifuged for 10 min at 3000*g*. The supernate was decanted immediately to avoid elution from the protein pellet. The supernates yielded no additional precipitate after a second addition of TCA. The pellets were dissolved in 2 ml of 2 *M* NaOH with gentle heating.

Aliquots were taken for determination of radioactivity in a liquid scintillation spectrometer[1] and for protein by the method of Lowry *et al.* (13), using human serum protein[2] as a standard. All samples were

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[1] Packard Instrument Company, LaGrange Illinois.
[2] Versatol, Warner-Chilcott Laboratories, Morris Plains, New Jersey.

recounted after addition of toluene-^{14}C[3] to correct for quenching. Binding was thereby defined as the moles of radioactive drug remaining firmly bound to protein after TCA precipitation. Calculations of mean, standard deviation, and Student's t test were by the usual formulae.

Results. Methodology. The binding process was immediate, for no difference in cpm/mg of protein was seen with any of the four major subcellular fractions after 5, 15, 30, or 60 min incubation.

The effects of changes in protein concentration on binding proved to be critical. Although no relationship was seen with concentrations above 1 mg/ml, with more dilute solutions binding (expressed per mg of protein) increased markedly. A fourfold increase occurred when the protein concentration was decreased eightfold, from 0.4 to 0.05 mg/ml. Though absolute cpm increased with increased amounts of protein, this increase was more than overcompensated and binding apparently decreased. Because of this relationship, unless protein concentrations are carefully monitored, comparisons or expressions of absolute degrees of binding are inappropriate.

Binding at 4° was 25% lower than at 37°C, with intermediate values found at room temperature. All further studies were carried out at 37°C.

Subcellular fractions. No difference in binding of pentobarbital was seen between the nuclei and mitochondria of newborn and adult rats (Fig. 1). Both fractions bound only moderate amounts as compared to the microsomal fraction. Moreover, this fraction bound significantly more (about twice as much) in the adult than the newborn. In the soluble fraction, which accounts for a major percentage of total binding by the cell, significantly more pentobarbital was bound by the newborn.

Nature of binding. Elution with isotonic sucrose from nondenatured proteins was studied to test reversibility. The percentage eluted during each exposure to a large excess of isotonic sucrose was uniform but differed consistently by fraction and age. Nuclei of adult animals retained about 40% of drug during each of three washes as opposed to 23% in newborn animals. No drug was retained by the mitochondrial fraction at any age. Retention by the microsomal fraction was intermediate in amount. Microsomes from adults had slightly higher values than newborns in one experiment, but this result was not reproducible.

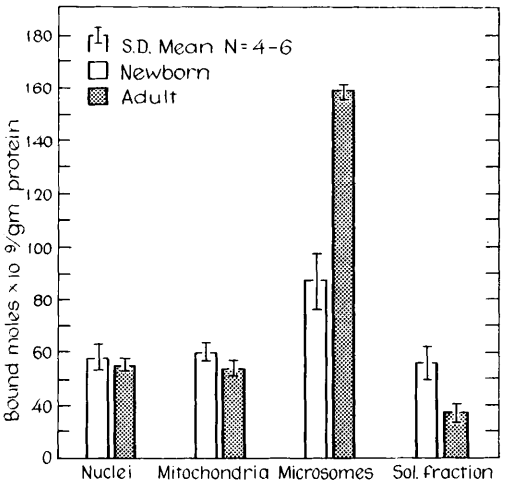

FIG. 1. Binding of pentobarbital-^{14}C by hepatic subcellular fractions. Incubation was at 37°C for 15 min after addition of 8.4×10^{-9} moles of pentobarbital-2-^{14}C.

Displacement by addition of a 1000-fold excess of unlabeled pentobarbital and incubation for an additional 15 min caused no significant decrease from the values shown in Fig. 1.

Differences between barbiturates. As seen in Table I, binding of pentobarbital and phenobarbital is equivalent but about tenfold greater than barbital.

Sex. No difference was found between the sexes with any drug or at either age studied (Table I), except for phenobarbital which seems to be bound in adult females more than by males ($p<0.05$). Due to the greater liver weight, binding per total organ was always greater in males.

Microsomal subfractions. Several investigations (14–16) have shown that drug metabolizing enzyme systems are preferentially localized in portions of the endoplasmic reticulum free of attached ribosomes (smooth or agran-

[3] Packard Instrument Company, LaGrange, Illinois.

TABLE I. Influence of Sex on Hepatic Microsomal Binding of Three Barbiturates.

Age	Drug	Microsomal binding (moles × 10^{-9}/gm of protein) Male Mean	Male SD	Female Mean	Female SD
Adult	Pentobarbital	266	23	275	36
	Phenobarbital	234	41	286[b]	44
	Barbital	23	4	22	3
42 days	Pentobarbital	1482	195	1522	88
	Phenobarbital	1825	89	1713	206
	Barbital	169	12	160	14

[a] Final concentration of each labeled drug was 1×10^{-5} *M*. Comparisons between ages are not appropriate because of differing protein concentrations (see text).

[b] Differs from male, $p < 0.05$.

ular). However, when the microsomal fractions were separated by ultracentrifugation in isooctane (12), binding was usually greater in the rough fraction (Table II). This phenomenon was observed using microsomal fractions from young rats with pentobarbital and barbital and with fractions from adult rats with barbital. No difference was found in the binding by smooth and rough vesicles obtained from infant animals.

Phenobarbital pretreatment. Administration of phenobarbital is known to cause proliferation of the endoplasmic reticulum, enhance the rate of metabolism of a number of drugs and increase the content of cytochrome P-450 (2,3,6). Nonetheless, no increase in microsomal binding of either of the barbiturates (per mg of protein) could be demonstrated after doses of phenobarbital (100 mg/kg for 5 days) which resulted in complete protection from a hypnotic dose of pentobarbital (30 mg/kg i.p.). In fact, binding appeared to be significantly decreased in the case of both pentobarbital and barbital. The problem was reexamined employing rough and smooth subfractions and pentobarbital binding was again found not to be increased above untreated control values.

Discussion. A major technical problem with investigations of this type lies in the separation of hepatic subcellular fractions. Although the procedures employed are those in common use, it is acknowledged that absolute uniformity of subcellular structural elements is not achieved. The major contamination, that of the nuclear fraction with membranes of the endoplasmic reticulum, should have little influence on the main result of this study. Dallner *et al.* (17) have shown that by 3 days after birth the cell population of the rat liver is comparable to that of the adult and differentiation of smooth and rough surfaced reticulum has been accomplished. Their electron microscopic studies revealed that the 105,000*g* pellet was only minimally contaminated by mitochondria and lysosomes, and

TABLE II. Binding of Pentobarbital and Barbital by Hepatic Microsomal Fractions from Rats of Different Ages.[a]

Age	Sex	Drug	Microsomal binding (moles × 10^{9}/gm of protein) Smooth Mean	Smooth SD	Rough Mean	Rough SD
3 days	Both	Pentobarbital	157	4	142	22
42 days	Both	Pentobarbital	649	140	1400[b]	193
Adult	F	Pentobarbital	815	93	839	132
42 days	Both	Barbital	67	15	283[b]	187
Adult	F	Barbital	109	21	300[b]	84

[a] Separation of smooth and rough vesicles was by ultracentrifugation in isooctane as described by Hallinan and Munro (12). Protein content (per gm of wet wt. of adult liver) of the three fractions so prepared was: rough, 10 mg; smooth, 5 mg; and ribosomes, 10 mg. The protein concentration of each fraction was brought to 0.3 mg/ml.

[b] Differs from smooth, $p < 0.001$.

was free of reticulocytic and nuclear fragments. Since our "newborn" rats were at least of this age it seems reasonable to assume that although losses may occur to other fractions the final microsomal pellet was reasonably homogeneous.

The increase in binding with temperature noted was contrary to the results obtained in two similar studies. Goldbaum and Smith (18) studying albumin binding of pentobarbital by ultrafiltration found no differences with temperatures of 4, 26, or 40°C. DeMoor *et al.* (19) found that corticoid binding, as determined by gel filtration, decreased with temperature elevation. It must be stressed that binding as determined in these studies was that withstanding TCA precipitation, presumably representing quite firm physicochemical bonding.

Results of the present study indicate that variation in drug–protein interaction *in vitro* parallels certain known differences in drug metabolism. For example, binding of the metabolizable barbiturates pentobarbital and phenobarbital was greater than for a nonmetabolized barbiturate, and binding was greater in adults than newborns.

However, increased binding per unit of protein did not account for other differences in metabolism, such as exists between sexes, microsomal fractions and following phenobarbital administration. Data on the latter two points was not as clear as desired and other techniques for separating smooth and rough vesicles (14,16) must be examined before preferential binding by the smooth fraction can be excluded. No technical reason could be found to explain the failure to demonstrate an increase in binding after phenobarbital pretreatment, despite its known effects of decreasing *in vivo* drug action, and increasing the level of cytochrome P-450 and maximum velocity (Vmax) of *in vitro* hydroxylation.

Summary. Studies of *in vitro* binding of barbiturates by rat hepatic subcellular fractions were performed to relate differences in substrate–protein interaction to alterations in drug metabolism. Binding of pentobarbital and phenobarbital by the microsomal fraction was tenfold greater than that of barbital. The microsomal fraction also exhibited an age dependent increase in binding per unit of protein, but no differences were found between sexes, or following phenobarbital pretreatment.

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