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# Binding of barbiturates to hepatic microsomes of the rat

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It has been recognized for more than a decade that certain drug substrates may bind to microsomes to a high enough degree that their free concentrations in the incubation medium may be lowered sufficiently to give misleading kinetic constants for the reaction under observation [1], yet microsomal drug-binding studies have been few and they have been limited largely to drugs that are known to bind very strongly to plasma proteins, such as imipramine [1–4] and 2-diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A) [1]. Recently, Bickel and Steele [4] published a comprehensive study which included Scatchard plots of the binding of chloropromazine, imipramine, methadone and phenylbutazone to rat hepatic microsomes. The current communication describes the binding of seven barbiturates to rat hepatic microsomes. Barbiturates were selected for this study because a great deal is known about their physical properties and their biotransformation by hepatic microsomes. The series includes three barbiturates (pentobarbital, phenobarbital and barbital) which were studied by Soyka [5] for their abilities to bind to hepatic microsomes of newborn and adult rats.

## MATERIALS AND METHODS

*Chemicals.* Secobarbital and amobarbital were obtained from Eli Lilly & Co., pentobarbital and hexobarbital from K & K Laboratories, phenobarbital and barbital from Merck Chemical Co. and aprobarbital from Hoffmann LaRoche, Inc. All were sodium salts; fresh solutions (50 mM) were prepared on the day of each experiment. All other chemicals were at least reagent grade.

*Preparation of microsomes.* Washed hepatic microsomes were prepared from male, albino Holtzman rats (200–250 g) as described previously [6]. The microsomal pellet was suspended in phosphate buffer (0.2 M, pH 7.4) to a final concentration of 2 or 4 mg protein/ml.

*Microsomal water.* Aliquots of microsomal suspensions containing from 2–20 mg of protein were centrifuged at 100,000 *g* for 60 min and the supernatants were removed by decantation. The pellets were dried to constant weight in a vacuum desiccator at room temperature over sodium hydroxide pellets. The difference between the wet and dry weights was used as the measure of the water content of the pellets. This was determined to be $6.9 \pm 0.5$ μl/mg of protein or $3.64 \pm 0.06$ μl/mg of dry microsomes (mean ± S. E.; n = 5). This is in reasonable agreement with Nilsson *et al.* [7], who found $3.30 \pm 0.08$ μl water/mg of dry microsomes.

*Binding of barbiturates to microsomes.* Various amounts of barbiturate (500–5000 nmoles) were mixed with 5.0 ml of microsomal suspension (2 or 4 mg protein/ml of phosphate buffer, 0.2 M, pH 7.4) using a vortex mixer, and allowed to stand at room temperature for 20 min. The suspensions were centrifuged at 100,000 *g* for 60 min. The pellets were extracted with 2.0 ml of 70% ethanol (v/v) using a vortex mixer. The extract was centrifuged at low speed, 1.5 ml of this supernatant fraction was mixed with 2.0 ml of 0.1 N NaOH solution, and the mixture was scanned between 280 and 230 nm against extracts prepared from microsomes which had not been exposed to barbiturates. Quantification was accomplished by peak height analysis with reference to 280 nm as the baseline. The accuracy of the method was assessed in each analysis by determining the barbiturate content of the 100,000 *g* supernatant fraction as well as that of the pellet. One ml of the 100,000 *g* supernatant fraction was mixed with 1.0 ml of 95% ethanol (v/v) and 2.0 ml of 0.1 N NaOH solution, and the barbiturate content of the mixture was determined as described for the microsomal pellet. Standard curves were established using both the 100,000 *g* supernatant fraction and the pellet to which known quantities of barbiturate had been added. Recoveries of added barbiturates from pellets and supernatant fractions were determined to be $99 \pm 2$ and $97 \pm 4$ per cent (mean ± S. E.), respectively, when microsomal suspensions of 2 and 4 mg protein/ml were used. Binding data were corrected as follows for the amount of barbiturate contained in the pellet water with the assumption that the concentration of barbiturate in this water was the same as that in the medium:

$$A = (B - C)/D, \text{ where}$$

$A$ = nmoles barbiturate/mg of pellet protein; $B$ = nmoles barbiturate in wet pellet, determined experimentally; $C$ = nmoles barbiturate in pellet water calculated by multiplying the nmoles barbiturate/ml of supernatant (determined experimentally after equilibrium was established) by the volume (ml) of water in the pellet; and $D$ = mg protein in pellet.

Spectral measurements were made with a Beckman model DB spectrophotometer.

*Microsomal protein.* The protein content of the microsomal suspensions was determined by the method of Lowry *et al.* [8].

*Statistical analysis.* Correlation analyses were made by the method of least squares linear regression. Correlation coefficients were examined for significance ($P \leq 0.05$) by a *t*-test reported by Goldstein [9].

### *Results and discussion*

Figures 1 and 2 show the binding of seven barbiturates (initial concentrations: 0.1, 0.5 and 1.0 mM) to microsomes (2 or 4 mg protein/ml). A comparison of Figs. 1 and 2 shows that the binding of each of the barbiturates, based on the amount of barbiturate bound per mg of protein, was approximately the same regardless of whether the medium contained 2 or 4 mg of microsomal protein/ml. These figures also show that binding was a linear function of the concentration of barbiturate throughout the range of concentrations employed. This also suggests that binding sites were probably not saturated at the highest initial concentration of barbiturates used (1.0 mM). Concentrations of barbiturate in the microsomes (nmoles/μl) relative to those in the medium were observed to range from about 2.5-fold (barbital to about 25-fold (secobarbital).

Using interpolated values from Figs. 1 and 2 for a barbiturate concentration of 0.5 mM, statistical analyses were performed to determine whether binding of barbiturates to microsomes might correlate with binding to bovine serum albumin, with corn oil–water partition coefficients, or with p*K*a values. The correlation coefficient ($r$) relative to binding of barbiturates to bovine serum albumin was determined to be 0.91 and 0.94 when the media contained 2 and 4 mg of microsomal protein/ml respectively (Fig. 3). Both values are significant at the $P < 0.01$ level. Correlation with oil–water partition was 0.83 and 0.85 when the concentration of microsomal protein was 2 and 4 mg/ml respectively (Fig. 4). These values are significant at the

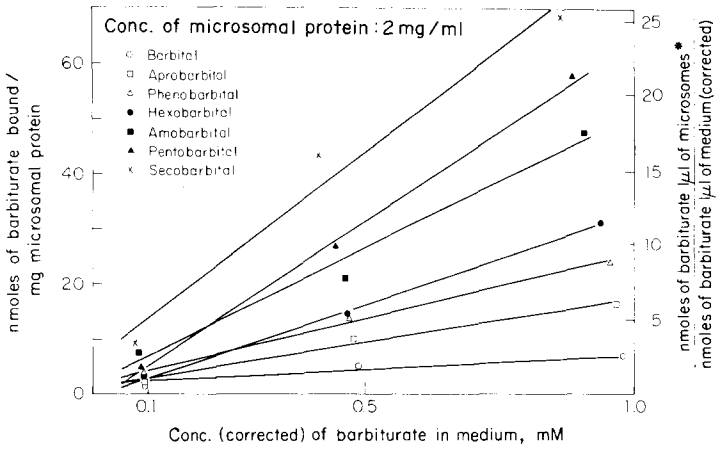


Fig. 1. Binding of barbiturates to hepatic microsomes (2 mg protein/ml). Concentrations of barbiturate were corrected to the concentration of barbiturate contained in the medium after equilibrium had been achieved between medium and microsomes as follows: $A = (B - aC)/1000$, where $A$ = concentration of barbiturate in medium after the addition of microsomes (mM); $B$ = nmoles barbiturate/ml of medium before the addition of microsomes; $C$ = nmoles barbiturate in microsomes represented by 1 mg of protein; and $a$ = mg of microsomal protein/ml of medium. All curves were drawn with the assistance of statistical analysis. The asterisk (*) indicates that the volume of microsomes relative to protein content was derived from the information that microsomes relative to protein content was derived from the information that microsomes contain 1.44 μl of intramicrosomal water/mg of dry weight of microsomes [7] and a phospholipid/protein ratio of 0.27 [10], and the assumptions that almost all of the microsome is composed of protein, lipid and water with specific gravities of 0, 0.9 and 1 respectively. In accordance with these observations and assumptions, the volume of microsomes represented by 1 mg of microsomal protein = $1.0 \times 1 + 0.27 \times 10/9 + 1.44 \times 1 = 2.74$ μl.

$P < 0.05$ level. No significant correlation was found between the p$K$a values of the barbiturates and binding to microsomes; the highest $r$ value was determined to be 0.26.

The binding of certain of the barbiturates to microsomes was of sufficient magnitude to raise the question as to whether or not binding might affect the measurement of apparent kinetic constants for the biotransformation of barbiturates by the microsomal mono-oxygenase system. The binding of secobarbital, pentobarbital and amobarbital to microsomes was great enough that falsely high apparent Michaelis constants would be derived if the concentration of microsomes was high.

The question is sometimes raised as to whether the concentration of a drug substrate in the medium of the incubation mixture is equal to, or merely relative to, the concentration of the substrate at the catalytic site in the microsome. The current study does not answer this question but demonstrates that the concentrations of different barbiturates—and by inference, other drug substances—may very greatly in the microsome. Concentrations of drug substrates at the catalytic site might be expected to vary accordingly. If, for the sake of discussion, it is assumed that each of the seven barbiturates would be biotransformed at the same true $V_{max}$ if capable of attaining the same molar concentration at the catalytic site, it could then be predicted that the apparent $V_{max}$ for a given barbiturate would be a function of its ability to accumulate at the catalytic site. In this case, the apparent $V_{max}$ values of 85, 341 and 91 moles substrate metabolized/mg of pro-

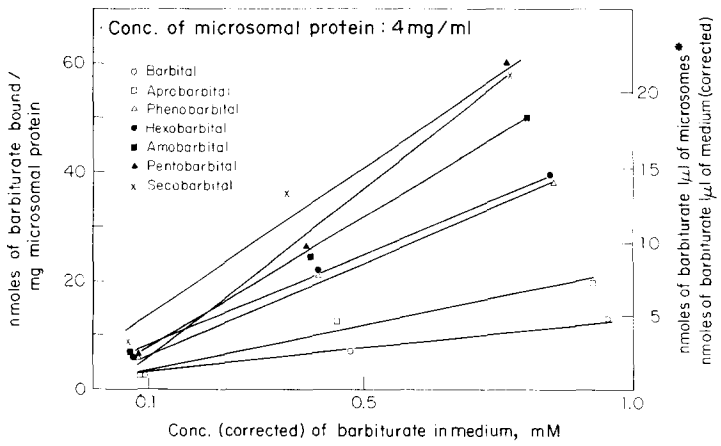


Fig. 2. Binding of barbiturates to hepatic microsomes (4 mg protein/ml). Relevant details are given in Fig. 1.

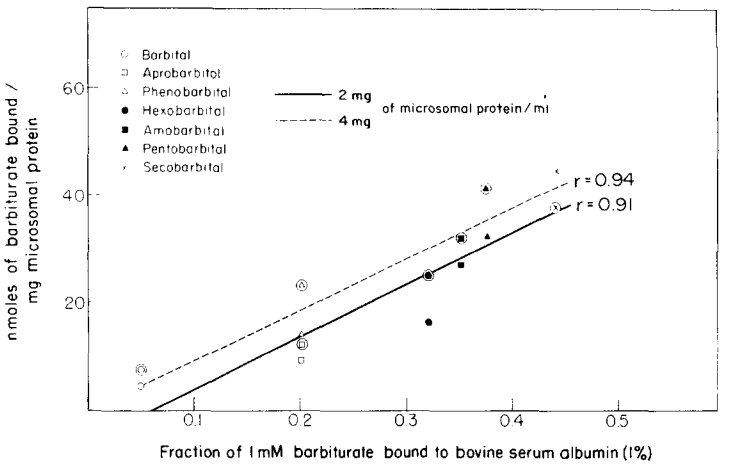


Fig. 3. Correlation of binding of barbiturates to microsomes with binding of barbiturates to bovine serum albumin. Values for binding of barbiturates to microsomes are interpolated values at the 0.5 mM concentration derived from Figs. 1 and 2. Values for binding of barbiturates to bovine serum albumin were taken from a publication by Goldbaum and Smith [11], except that for hexobarbital, which was determined in our laboratory by their method. Uncircled and circled symbols designate values obtained with 2 and 4 mg of microsomal protein/ml respectively.

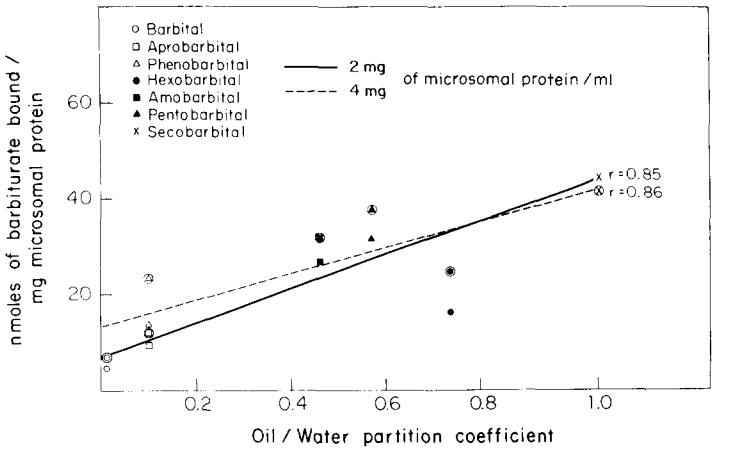


Fig. 4. Correlation of binding of barbiturates to microsomes with corn oil/phosphate buffer (pH 7.4) partition coefficients. Values for binding of barbiturates to microsomes are interpolated values at the 0.5 mM concentration derived from Figs 1 and 2. Partition coefficients were taken from a publication by Jansson *et al.* [12]. Uncircled and circled symbols designate values obtained with 2 and 4 mg of microsomal protein/ml respectively.

tein/hr for anobarbital, hexobarbital and pentobarbital, respectively [6], might be expected to relate to their microsomal binding capacity. In fact, such a relationship does not exist; of the three barbiturates, hexobarbital had the highest $V_{max}$, but the lowest binding capacity.

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# Correlation between gastric protein kinase and secretion in the pylorus ligated rat

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Protein kinase has been shown, in many tissues, to mediate some of the biological effects of cyclic nucleotides [1]. Investigators have implicated cyclic AMP (cAMP) and/or cyclic GMP (cGMP) in the regulation of gastric secretion [2, 3]; however, evidence suggesting the mediation of this physiological effect by cAMP and/or cGMP activated protein kinase activity *in vivo* has not been reported. The isolation and characterization of protein kinase in the rabbit gastric mucosa [4, 5] raised a possibility that these enzymes mediate the effects of cyclic nucleotides on gastric secretion.

We have undertaken an investigation of the effects of certain anti-secretory agents on gastric protein kinase activity in order to demonstrate a possible relationship between enzyme activity and acid secretory levels. For this investigation we have selected the pylorus ligated rat, commonly used in gastric secretory studies.

Male Sprague-Dawley rats (body wt 160–180 g) were fasted for 18 hr with free access to water until compound administration. Thirty minutes after compound treatment the pylorus was ligated. Two hr following pylorus ligation the animals were sacrificed by cervical dislocation and their stomachs removed and emptied for analysis of gastric juice volume and acidity. The stomachs were placed in ice-cold saline prior to removal of the mucosa. The mucosal layer was scraped and pooled (two animals/group) and then homogenized with a glass-to-glass homogenizer in 20 vol. of 0.25 M sucrose containing 5 mM $MgCl_2$ and 25 mM KCl. Homogenates were centrifuged at 27,000 *g* for 15 min and the resulting clear supernatant was used as the enzyme source. Protein kinase activity was determined according to the procedures of Miyamoto *et al.* [6] and Hiestand *et al.* [7] in a total vol. of 0.2 ml. The standard incubation mixture contained DL-α-glycerophosphate buffer (pH 7.5), 50 μM; protamine sulfate, 40 μg; ($\gamma$-$^{32}$P) ATP, 10 μM; $MgCl_2$, 10 mM; NaF, 10 mM; theophylline, 4 mM; dithiothreitol, 10 mM; mucosal enzyme, 20 μg; and cyclic nucleotide as indicated. Trichloroacetic acid (TCA)-precipitable proteins were collected on Millipore membranes (Type HA, 0.45 μm) and the incorporation of pMoles of $^{32}$P into these proteins was calculated after enzyme blank subtraction. For this crude enzyme extract we determined the optimum pH, optimum cyclic nucleotide concentration, time course and $K_m$. Finally, the effects of the anti-secretory agents, 2-pyridylthioacetamide (CMN-131) [8], metiamide [9] and propantheline [10] on this enzyme were determined *in vivo* with the pylorus ligated rat preparation.

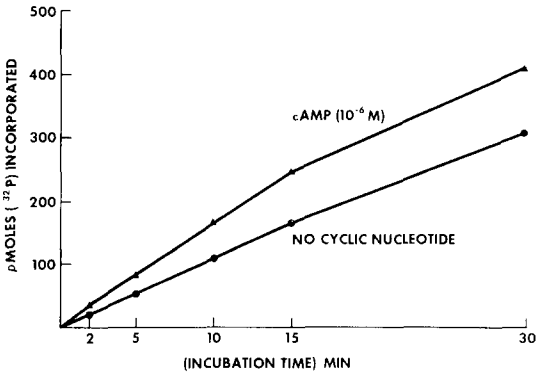


Fig. 1. Protein kinase time response. ATP = 10 μm.

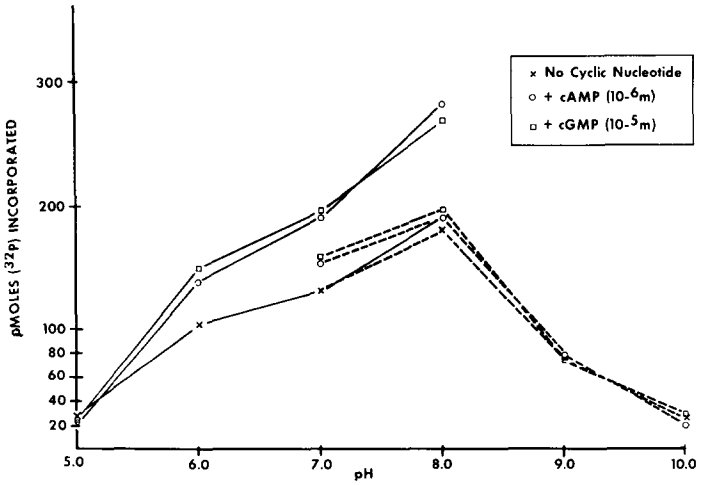


Fig. 2. Activity of rat gastric mucosal protein kinase as a function of pH, in 0.05 M DL-α-glycerophosphate buffer (solid lines) and in 0.05 M Tris-HCl buffer (hatched lines). ATP = 100 μM (2.5 × $K_m$), $t$ = 15 min.