STRUCTURE OF CHEMICAL COMPOUNDS, METHODS OF ANALYSIS AND PROCESS CONTROL

ENZYME – SUBSTRATE COMPLEXES OF CYTOCHROME P-450 WITH SOME BIOLOGICALLY ACTIVE COMPOUNDS STUDIED BY ELECTRONIC ABSORPTION SPECTROSCOPY

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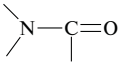
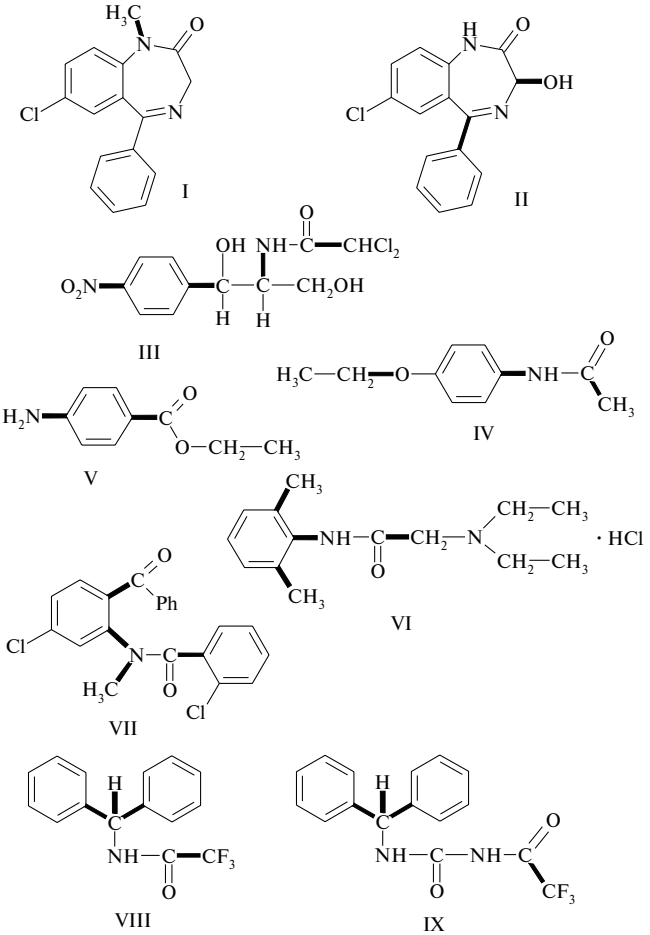
Previously [1 – 4], we studied the dissociation constants K_S of complexes of a large group of nitrogen-containing compounds with cytochrome P-450 of liver microsomes by spectroscopic techniques. Most of the ligands studied in [1 – 3], representing derivatives of urea, dibenzazepinone, barbituric acid, and some other heterocyclic compounds, contain peptide groups of the  type. The peptide fragment also enters into the compositions of many well-known drugs.

In this study, we determined the K_S values for a group of such drugs, including diazepam (I), nozepam (II), chloramphenicol (III), phenacetin (IV), anesthesin (V), lidocaine (VI), and 2-benzylacetanilide (VII) (cliche) (the bioconversion of which proceeds with the participation of some isoforms of cytochrome P-450 [5]), as well as of two compounds, N-trifluoroacetylbenzhydrylamine (VIII) and N-benzhydryl-N′-trifluoroacetylurea (IX), possessing anticonvulsant activity [6, 7].

The pK_S values for compounds I – IX were calculated using QSAR relationships [8] derived by the method of frontal polyhedra (FP) [9, 10]. A comparative analysis of the experimental and calculated values, together with analogous data obtained previously for some derivatives of diphenylamine, benzimidazole, benzotriazole, and phenoxazine [4], allowed us to evaluate the ability of the FP method to predict the dissociation constants of the enzyme – substrate complexes of microsomal cytochrome P-450.



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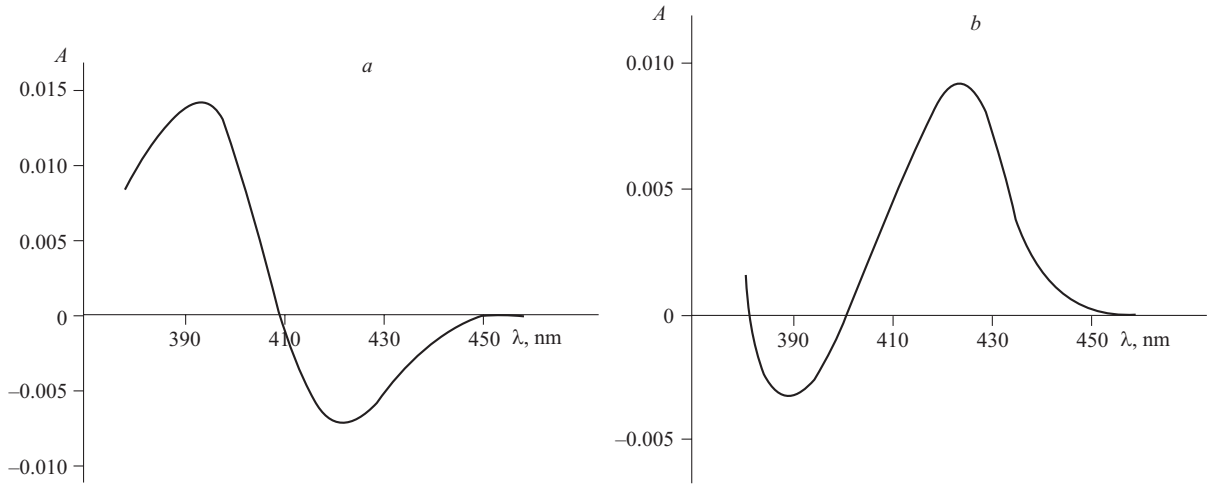

Fig. 1. Differential electronic absorption spectra of (*a*) lidocaine (compound VI) and (*b*) diazepam (compound I) for [VI] = 2.388×10^{-3} M, [I] = 0.565×10^{-3} M, and [P-450] = 4.5 nmole/ml (optical cell thickness, 1.0 cm).

EXPERIMENTAL PART

The experiments were performed on a group of male Wistar rats weighing 180 – 200 g, injected intraperitoneally with sodium phenobarbital (Merck) in a single daily dose of 80 mg/kg over a period of three days. The animals were decapitated 48 h after the last injection and the microsomal liver fraction was isolated by the differential centrifugation technique [11]. The content of cytochrome P-450 was determined according to Omura and Sato [12], and the protein concentration, by the method of Lowry et al. [13, 14].

Compounds I – VII were represented by pharmacopoeial preparations; the trifluoroacetyl derivatives VIII and IX were synthesized as described in [6, 7].

The electronic absorption spectra were measured at room temperature on a Perkin-Elmer Model 124 spectrophotometer. Immediately prior to the photometric measurements, the suspension of microsomes was diluted with 0.1 M phosphate buffer (pH 7.4); aliquots of this solution were placed into optical cells. The cytochrome P-450 concentration in the sample was 4.5 nmole/ml and the protein concentration, 2.3 mg/ml. During titration, solutions of compounds I – IX in DMSO (freshly distilled immediately before use) were added to the microsomal suspension in the working cell and equal volumes of DMSO were added to the reference cell. The differential electronic absorption spectra were measured at room temperature in a wavelength range from 370 to 470 nm. The substrate concentration in the working cell varied within $(0.07 – 4.21) \times 10^{-3}$ mole/liter. The DMSO content in the working cell during the microsomal suspension titration with the substrate solutions did not exceed 7.6%.

The apparent dissociation constants K_S were determined by the least squares method using linear plots constructed in Lineweaver – Burk coordinates.

With the purpose of predicting the K_S values, the molecular geometry of compounds I – IX were modeled by the molecular mechanics method using an ASTRA program package [15] with a parametrization described in [16]. The resulting three-dimensional models were analyzed by a modified FP method [10] involving subdivision of the molecules into "rigid" and "labile" fragments. The rigid fragments are indicated (bold bonds) in the structural scheme. The class of rigid fragments included all atomic groups except the one-, two-, and three–atomic (H, C=O, OH, CH_2, NO_2) ones and submolecules containing internal rotational degrees of freedom (such as fragments containing a hydroxy group in compound III and the $–CH_2N<$ group in compound VI). The optimum superimpositions for the FP model [9] were determined using a special procedure developed previously for analysis of the structure – pK_S relationship [8]. The optimum superimpositions determined for each of the substrates (I – IX)

TABLE 1. Characteristics of Enzyme – Substrate Complexes between Compounds I – IX and Cytochrome P-450

Compound	Type of spectral changes* (λ_{min}, λ_{max}, nm)	K_S,** mole/liter	*N*
I	*II* (389, 423)	$6.0\ (31) \times 10^{-4}$	6
II	*II* (397, 424)	$1.70\ (5) \times 10^{-4}$	5
III	*II* (392, 423)	$1.34\ (18) \times 10^{-4}$	4
IV	*II* (383, 424)	$2.14\ (7) \times 10^{-5}$	8
V	*II* (389, 423)	$1.68\ (21) \times 10^{-5}$	8
VI	*I* (422, 393)	$4.02\ (7) \times 10^{-4}$	7
VII	*I* (422, 394)	$1.07\ (6) \times 10^{-5}$	4
VIII	*II* (392, 423)	$1.77\ (23) \times 10^{-5}$	4
IX	*II* (386, 425)	$1.97\ (47) \times 10^{-5}$	5

* The positions of minima and maxima are determined with an error of 1 – 2 nm.

** Values in parentheses indicate mean-square deviations in the last digit.

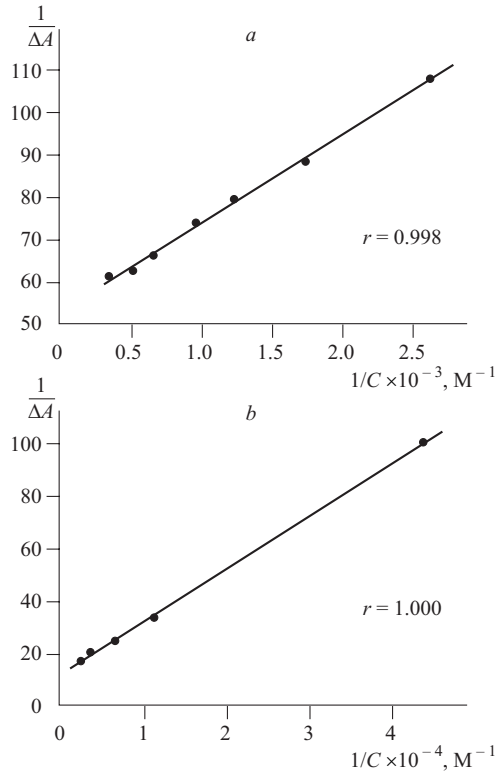

Fig. 2. Lineweaver – Burk plots of the amplitude of spectral changes A versus substrate concentration C for the complexes of substrates (a) II and (b) VI with cytochrome P-450 4.5 nmole/ml (optical cell thickness, 1.0 cm).

TABLE 2. Experimental and Calculated pK_S Values for Cytochrome P-450 Complexes with Various Substrates*

Compound	pK_s	pK'_S	$pK'_S - pK_S$
I	3.221	3.635	– 0.414
II	3.770	4.079	– 0.309
III	3.873	3.489	0.384
IV	4.670	4.618	0.052
V	3.775	3.955	– 0.180
VI	3.396	3.567	– 0.171
VII	4.971	4.551	0.420
VIII	4.752	4.777	– 0.025
IX	4.706	5.286	– 0.580
X	4.496	4.378	0.118
XI	4.939	4.628	0.311
XII	5.010	4.443	0.567
XIII	3.785	4.382	– 0.597
XIV	5.257	5.182	– 0.075
XV	3.963	4.351	– 0.388
XVI	5.299	5.286	0.013
XVII	5.300	5.534	– 0.234
XVIII	5.658	5.515	0.143

* The pK_S and pK'_S values for compounds X – XVIII are taken from [4].

were used to calculate the pK_S values based on the QSAR model [8].

RESULTS AND DISCUSSION

It was established that titration of microsomal suspensions with solutions of substrates VI and VII leads to changes of type *I* in the differential absorption spectra, while titration with compounds I – V, VIII, and IX gives rise to spectral changes of type *II* [17, 18] (Table 1). The differential absorption spectra of the complexes exhibit minima at λ_{min} = 415 – 422 (compounds VI, VII) and 383 – 397 nm (I – V, VIII, IX) and maxima at λ_{max} = 393 – 394 (VI, VII) and 423 – 425 nm (I – V, VIII, IX) (see typical examples in Fig. 1).

It was suggested [1 – 4, 19] that the spectral changes of type *II* are related to the interaction between substrate ligands, containing nitrogen atoms with unshared electron pairs (UEPs), and Fe^{3+} ions in the heme fragment of cytochrome P-450. From this standpoint, the identity of nitrogen-containing compounds I – V, VIII, and IX as substrates of type *II* is readily explained, since these molecules contain sterically accessible UEPs with a degree of localization sufficient to provide for interaction with heme iron. In this context, it should be noted that the nitrogen-containing ligands involved in complexation with cytochrome P-450 give rise to spectral changes of type *II* only in the case of considerable UEP delocalization (e.g., upon simultaneous conjugation with two carbonyl groups [3] or sterical screening of the nitrogen atom [4]). Apparently, the presence of ortho-methyl groups and ethyl substituents makes the UEP of molecule VI (type *I* substrate) insufficiently sterically accessible for complexation with the Fe^{3+} ion. In compound VII, the atom of nitrogen is also surrounded by three substituents and its UEP enters into the common conjugation system of the molecule.

The linear relationships, plotted in double inverse logarithmic Lineweaver – Burk coordinates for determining the K_S values of the enzyme – substrate complexes, have correlation coefficients of not less than 0.981. Figure 2 shows typical Lineweaver – Burk plots and Table 1 gives values of the dissociation constants K_S and the numbers of experimental points N used for their determination.

Judging by the relatively high K_S values, substrates I – IX possess a not large affinity to cytochrome P-450 of phenobarbital-induced liver microsomes. For comparison, we can mention some derivatives of diphenic acid [2], benzhydrylurea [1], and benzimidazole and benzotriazole [4] possessing K_S on the order of 10^{-6} – 10^{-7} M.

Since the compounds studied in this work belong to various structural groups, it is difficult to reveal visually some structural elements that may account for the stability of their enzyme – substrate complexes with cytochrome P-450. Previously [8], we used the FP method to construct a QSAR model relating the chemical structure to K_S values for a

group of 36 substrates representing structurally different ligands of type *II*. It was expedient to use the results of experimental determination of the K_S values (Table 1) for additional verification of the predicting ability of the above QSAR model. Table 2 summarizes the pK_S values experimentally determined for the cytochrome P-450 complexes with compounds I – IX in comparison with the pK'_S values determined by calculation based on the QSAR relationships [8].

In addition, we have analyzed analogous data reported previously [4] for benzimidazole (X), 1-allylbenzimidazole (XI), 2-(*o*-fluorophenyl)benzimidazole (XII), benzotriazole (XIII), *cis*-1-propenylbenzotriazole (XIV), and diphenylamine (XV) (substrates of type *II*), as well as for N-allyldiphenylamine (XVI), *cis*-10-propenylphenoxazine (XVII), and 10-allylphenoxazine (XVIII) (substrates of type *I*). The pK'_S of compound XVI – XVIII were calculated based on a special QSAR model [8] constructed using a teaching set of 15 ligands producing spectral changes of type *I* upon forming complexes with cytochrome P-450.

A comparison of the measured and calculated values presented in Table 2 shows a good agreement between theory and experiment (Fig. 3). The rms deviation of pK'_S from pK_S is 0.334, which is comparable with the values $S = 0.324$ and 0.333 determined using the adequacy dispersions of the QSAR models for substrates of types *I* and *II*, respectively [8]. The quality of prognosis provided by the R_c^2 [2, 20] models is frequently evaluated by the value

$$R_c^2 = 1 - \frac{\sum_{i \in C} (pK_{S,i} - pK'_{S,i})^2}{\sum_{i \in C} (pK_{S,i} - \overline{pK}_{S,i})^2},$$

where $pK_{S,i}$ and $pK'_{S,i}$ are the experimental and calculated pK_S values for complexes with the *i*th ligand and $\overline{pK}_S$ is the average pK_S value determined for substrates of the teaching set employed for $pK'_{S,i}$ determination. Determined from the data from Table 2, the R_c^2 value for compounds I – XVIII amounts to 0.786.

Thus, the quantitative relationships between structure and properties established in [8] allow sufficiently reliable estimation of the dissociation constants for enzyme – substrate complexes of microsomal cytochrome P-450.

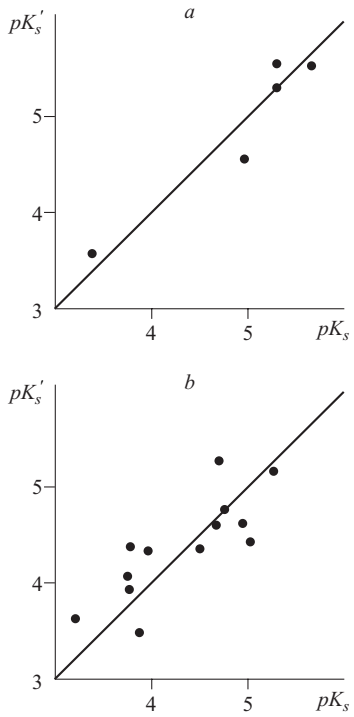

Fig. 3. Correlation between experimental and calculated pK_S values for a control set of substrates of (*a*) type *I* (compounds VI, VII, XVI, XVII, XVIII) and (*b*) type *II* (compounds I – V, VIII – XV).

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