SPECTRAL CHANGES IN CYTOCHROME P-450 OF RAT LIVER MICROSOMES DURING INTERACTION WITH PHENAZEPAM AND ITS 3-HYDROXY METABOLITE

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The formation of a complex with the oxidized form of cytochrome P-450 is the first and essential stage of oxidation of drugs. Changes in spectral characteristics of the hemoprotein arising under these circumstances provide essential information for the solution of problems connected with the mechanism of their enzymic hydroxylation.

This paper gives the characteristics of spectral changes in cytochrome P-450 of liver microsomes during interaction with phenazepam and its 3-hydroxy metabolite. The atomic charges of the compounds are compared with kinetic parameters characterizing the benzodiazepine–hemoprotein complex.

EXPERIMENTAL METHOD

Male Wistar rats weighing 150-180 g were used. The animals were given intraperitoneal injections of phenobarbital (80 mg/kg) and 3-methylcholanthrene (40 mg/kg), dissolved in sunflower oil, daily for 3 days. Microsomes were isolated from the rat liver by ultracentrifugation of the postmitochondrial fraction in medium containing 1.15% KCl and 40 mM Tris-HCl buffer, pH 7.4, in a "Beckman" ultracentrifuge at 105,000g. Spectral changes of complexes of cytochrome P-450 with 1,4-benzodiazepines were recorded on an "Aminco" spectrophotometer. The microsomes were diluted with 40 mM Tris-HCl buffer, pH 7.4, to a protein concentration of 2.6 mg/ml. The substrates were dissolved in methanol to a concentration of 30 mM and added to 3 ml of a suspension of microsomes. An equal quantity of methanol was added to the control cuvette. After each addition the difference between absorption at 390 and 420 mm was recorded. The measurements were made at room temperature [1].

Atomic charges of the 1,4-benzodiazepine molecules were calculated by Del Rey's method realized by the "Sigma" program, using parameters in [3], on the M-220 M computer.

EXPERIMENTAL RESULTS

In albino rat liver microsomes phenazepam (I) is oxidized into a 3-hydroxy metabolite (II).

This reaction is catalyzed by mono-oxygenases, containing cytochrome P-450 as the terminal oxidase.

Cytochrome P-450 of the liver microsomes of rats receiving injections of phenobarbital, on interaction with I, gave a maximum of absorption at 412 nm and a minimum at 384 nm, with isobestic point at 400 nm.

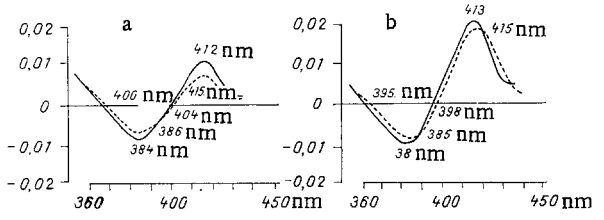

Fig. 1. Differential spectra of liver microsomes of rats receiving phenobartital (a) or 3-methylcholanthrene (b), observed during titration with phenazepam (continuous line) and its 3-hydroxy metabolite (broken line).

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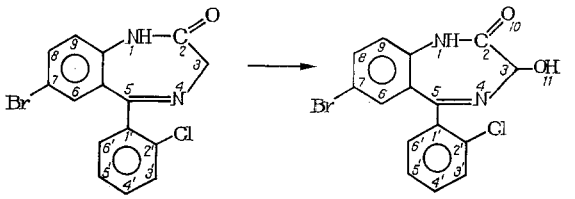
TABLE 1. Differential Spectra of Rat Liver Microsomes during Introduction of Cytochrome P-450 by Phenobarbital (PB) and 3-Methylcholanthrene (MC) on Addition of Phenazepam (I) and Its 3-Hydroxy Metabolite (II) (cytochrome P-450 concentration 1.30 nmole/mg protein in the case of induction by PB, 0.98 nmole/mg protein for induction by MC)

Experimental conditions	PB		MC	
	I	II	I	II
Concentration range, μmoles	20±300	20—300	40—400	40—250
ΔD_{max}, nmoles cytochrome P-450	0,0014	0,0016	0,0006	0,0020
K_S, M	$0{,}76\cdot10^{-4}$	$1{,}25\cdot10^{-4}$	$1{,}0\cdot10^{-3}$	$1{,}0\cdot10^{-4}$
λ_{min}, nm	384	386	383	385
λ_{max}, nm	412	415	413	415



I II

For II the corresponding values were 415, 386, and 404 nm (Fig. 1a). In the case of the hemoprotein binding spectrum in the liver of rats receiving 3-methylcholanthrene, a similar picture was observed, but with a small shift of the characteristic points (Fig. 1).

The character of interaction of the hemoproteins with I and II corresponded to the second, or aniline, type of substrate binding. It takes place as a result of direct addition of the substrate molecule to the heme group of cytochrome P-450 [1, 6]. Measurement of the amplitude of the spectral changes of the hemoprotein—benzodiazepine complex showed that they depend on substrate concentration. Titration of liver microsomes of rats receiving phenobarbital with benzodiazepines showed that the enzyme is saturated by phenazepam in a concentration of 300 μM and by its 3-hydroxy metabolite in a concentration of 200 μM.

Values of K_S and ΔD_{max} for I and II, determined graphically by the method of Lineweaver and Burk, differed considerably (Table 1).

During induction of the liver mono-oxygenases of the animals by 3-methylcholanthrene saturation of the hemoprotein occurred with I in a concentration of about 400 μM and II about 100 μM. Meanwhile the optical properties of the complex changed, with a reduction in the value of ΔD_{max} for I by about half and a very small increase in this parametre for II. Corresponding differences in the optical properties of the complex of the hemoprotein with I and II observed in this cse may be due to an increase in the content of cytochrome P-448, which has substrate properties and spectral characteristics different from those of cytochrome P-450 [4], under the influence of 3-methylcholanthrene.

On the whole, for both I and II, low values of K_S were obtained, such as are characteristic for most of the 1,4-benzodiazepines [1] and they did not coincide with the values of K_m [2], evidence that these substrates bind with the hemoprotein at two sites — heme and apoenzyme. During interaction with the first center, spectral changes of the second type appeared, whereas with the other (substrate formation), the changes were those of the enzyme complex responsible for oxidative transformation of I and II. Intensive oxidative metabolism of phenazepam, leading to the formation of metabolites hydroxylated at different positions of the molecule is evidence in support of the second. In fact, for dimethyldiazepam, the first type of spectral binding with microsomes of intact rats was found at low concentrations of this substrate also, in the case of liver microsomes of rats receiving phenobarbital. In higher substrate concentrations, and also during induction with 3-methylcholanthrene, conversion into the second type of spectral binding took place [7].

TABLE 2. Charges on Atoms of Benzodiazepine Ring of Phenazepam (I) and Its 3-Hydroxy Metabolite (II)

Atom No.	Substance		Difference, atomic units
	I	II	
1	—0,3298	—0,3287	0,0011
2	0,1177	0,1283	0,0106
3	0,0302	0,1871	0,1569
4	—0,2759	—0,2640	0,0119
5	0,1367	0,1384	0,0017
6	—0,0274	—0,0274	—
7	0,0159	0,0159	—
8	—0,0268	—0,0268	—
9	0,0461	—0,0461	—
10	—0,0719	—0,0698	0,0021
11	—	—0,4458	—
1′	0,0373	0,0374	0,0001
2′	0,1031	0,1031	—
3′	—0,0279	—0,0279	—
4′	—0,0499	—0,0499	—
5′	—0,0523	—0,0523	—
6′	—0,0508	—0,0508	—

This change in the type of spectral binding may take place during induction of hemoproteins on account of an increase in the number of sites responsible for the second type of spectral changes.

Under these circumstances the actual structure of the benzodiazepine molecule, which is characterized by the possession of several different stereochemical sites (benzene ring, condensed with the heterocyclic ring, the heteroring itself, and the free benzene ring), will be of great importance. This structure enables the benzodiazepine molecule to interact both with different binding sites and also with modifications of the hemoprotein, the ratio between which will determine the type of interaction.

The causes of differences in spectral characteristics (K_S, ΔD) of the complexes, and also the shift of the maximum and minimum points, caused by phenazepam and its 3-hydroxy metabolite, may be determined by the electronic structure of these compounds

An important role in the formation of such a spectrum for substrates with the second binding type has been shown to be played by nitrogen atoms, which interact directly by their unshared electron pair with heme iron. Such interaction is possible on displacement of protein groups coordinated with the iron atom, possibly by the mercaptide group of cysteine and the imidazole group of the histidine residue [6].

For this purpose the electronic structure of the molecules of the substrates used, in which electron densities on different atoms were determined, was studied.

Analysis of the charge values, represented in atomic units, showed that in 3-hydroxyphenazepam the distribution of electron density on the heteroring differs from that observed for phenazepam (Table 2). For instance, under the influence of the hydroxy group attached to the C_3 carbon atom there is a shift of electron density toward the oxygen atom, thus conferring on the latter a high negative charge (—0.4458). One result of this is a corresponding decrease in the charge on the nitrogen atoms (N_1 by 0.0011 atomic unit, N_4 by 0.0119 atomic unit). An increase in the relative positive charge by 0.0106, 0.1569, and 0.0017 atomic unit takes place by the C_2, C_3, and C_5 carbon atoms respectively.

The influence of the hydroxy group does not extend to bromine-containing and chlorophenyl aromatic rings. This indicates that the latter do not take part in the formation of the differences observed between these substrates. A reduction in the negative charge by 0.0021 atomic unit also is observed for the oxygen atom attached to the C_2 carbon atom.

It can be postulated on the basis of these data that shifts of maximum and mimimum points observed for II into the long-wave region, and also differences in the kinetic parameters of binding compared with phenazepam may be caused by the influence of the hydroxy group, reducing the electron density of the heteroring, in particular, on nitrogen atoms (N_1 and N_4). Changes in the state of the electrons of heme iron may arise under these circumstances, affecting in turn the axial ligands on the apoenzyme. This may cause corresponding conformational changes in the macromolecule. The possibility cannot be ruled out that hydroxyl group II may interact with polar groups of adjacent amino-acid residues, thus modifying the posi-

tion of the plane of the porphyrin ring, as a result of which a change in interaction between porphyrin and the amino-acid skeleton of the molecule will take place [5].

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THE ANTISTRESSOR EFFECT OF D-Ala^2-Leu^5-Arg^6-ENKEPHALIN

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Besides known biologically active substances, the opioid neuropeptides, whose level in the body rises regularly during exposure to extremal factors, may behave as endogenous modulators of stress [6, 7]. Accordingly the aim of this investigation was to study the effect of enkephalins on a combination of nonspecific morphological changes (Selye's triad) arising in animals and the glucocorticoid level in stress.

EXPERIMENTAL METHOD

Experiments were carried out on 50 noninbred male albino rats weighing 100-120 g and on 40 noninbred albino mice wieghing 18-20 g. Stress was induced by suspending the animals for 18 h by the neck fold. All the animals were divided into groups (10 in each group): 1) intact animals; 2) stress control; 3-5) before the beginning of exposure to stress, the synthetic arginine-containing hexapeptide analog of Leu-enkephalin (D-Ala^2-Leu^5-Arg^6-enkephalin) in various doses or a preparation of extract of eleutherococcus (1 ml/kg, perorally) was administered to the animals; the latter is known to be one of the most effective adaptogens [2, 3]. The animals were killed in accordance with the "Rules for the conduct of research using experimental animals" approved by the Ministry of Health of the USSR. The weight of the adrenals, spleen, and thymus was determined in milligrams, and the number of ulcers formed on the gastric mucosa was counted. Antistressor activity of the preparations was determined using the six-point scale for evaluation of stress suggested by Lobryakov [4]. It was considered that the preparation possessed antistressor activity if the difference in the total number of points between the stress control and the experimental groups exceeded or was equal to 2. To count the points for integral assessment of the degree of stress in an animal, values of the mean weight of the seperate organs in the groups were calculated per 100 g body weight of rat or per 20 g body weight of mouse,and changes in the parameters were expressed as percentages of the normal [4]. The 11-hydroxycorticosteroid (11-HCS) concentration in samples of blood plasma from the rats was determined by a fluorometric method [5] on a Hitachi (Japan) spectrofluorometer, and the immunoreactive cortisol level was determined by radioimmunoassay with the kit from CEA-IRE-Sorin (France) on a Tracor gamma-spectrometer (USA). The

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