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Stereospecificity of Esterases Hydrolyzing Oxazepam Acetate

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Abstract □ Esterases hydrolyzing the racemic acetate ester of the centrally acting drug oxazepam in mice were examined. Radiolabeled ester administered intravenously was hydrolyzed rapidly in the liver, kidneys, and brain. The distribution of the enzyme activity of liver and brain subcellular fractions was measured. Kinetic data and structure investigation of partially hydrolyzed racemic ester pointed to the stereoselectivity of liver and brain esterases. The preferred hydrolysis of the (*R*)-(−)-isomer in liver homogenates was attributed mainly to microsomal enzymes, while that of the (*S*)-(+)-isomer in brain was considered to be due to the mitochondrial fraction. This phenomenon was a common property of all species tested.

Keyphrases □ Oxazepam acetate—hydrolysis *in vivo* and *in vitro*, stereospecificity of esterases determined □ Hydrolysis—oxazepam acetate *in vivo* and *in vitro*, stereospecificity of esterases determined □ Stereospecificity—esterases hydrolyzing oxazepam acetate *in vivo* and *in vitro* □ Esterases—hydrolyzing oxazepam acetate *in vivo* and *in vitro*, stereospecificity determined □ Tranquilizers—oxazepam, hydrolysis of acetate ester *in vivo* and *in vitro*, stereospecificity of esterases determined □ Enzymes—esterases hydrolyzing oxazepam acetate *in vivo* and *in vitro*, stereospecificity determined

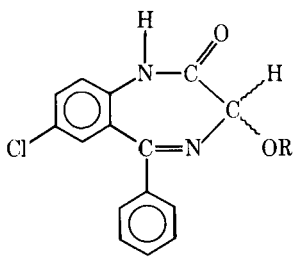
The 1,4-benzodiazepines are effective sedatives and anticonvulsants. The 3-substituted derivatives are less active (1), with the exception of oxazepam (I) and its hydrolyzable compounds. Esters as prodrugs may modify and prolong the pharmacological action of a drug (2), but the succinate half-ester (III) is one of the few derivatives of I that has been tested this way (3). Because of its asymmetric structure, there were stereoselective differences in biological activity (4, 5), ester hydrolysis (6), and serum albumin binding (7, 8).

This paper reports a study of *in vivo* and *in vitro* hydrolysis of oxazepam acetate (II), which has nearly the same pharmacological properties as I (1). Since it hydrolyzes rapidly, its decomposition site was examined down to the subcellular level, and attention was focused on stereospecificity.

EXPERIMENTAL

Animals—Male albino mice, 20–25 g, were used.

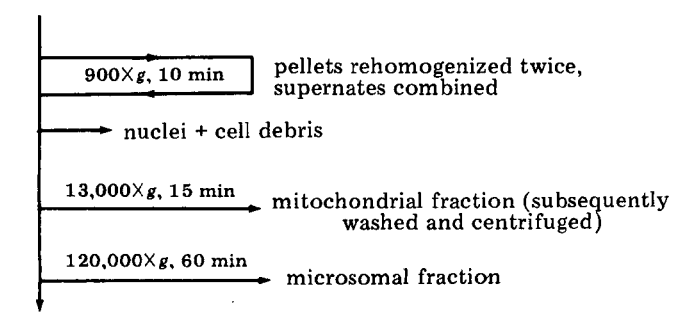
Substrates—Oxazepam acetate was obtained by rearrangement of 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepine-2-one 4-oxide with acetic anhydride (9). The specific activity of the 2-^{14}C-compound was 3.15 mCi/mmole. β-Naphthyl acetate and physostigmine sulfate were used as received[1].



I: R = H
II: R = $COCH_3$
III: R = $COCH_2CH_2COOH$

Pharmacokinetics *In Vivo*—A dose of 11 mg of 2-^{14}C-II/kg iv was administered to mice in 70% aqueous dimethyl sulfoxide (2.5 ml/kg). After decapitation, the brain and blood were immediately homogenized in cold methanol containing nonlabeled I and II in a 50-fold excess. After 5 min of shaking (isotope exchange equilibrium was attained), the samples were centrifuged, and the supernate was then spotted on silica gel thin-layer plates. The developing system was benzene–ether–ethanol (5:5:0.7). The radioactivity of the spots suspended with a gelling agent[2] was measured on a liquid scintillation spectrometer[3].

Tissue Fractionation—The liver and brain were homogenized in 0.25 and 0.32 *M* sucrose, respectively. Fractionation was accomplished by ultracentrifugation[4] with a combination of previous methods (10, 11) according to Scheme I. All fractions were then resuspended in 0.25 and



Scheme I

[1] Fluka and Calbiochem.
[2] Aerosil 380, Degussa, Frankfurt am Main, West Germany.
[3] Packard type 3003.
[4] Janetzky type VAC-601.

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Table I—Esterase Activity of Tissues for Oxazepam Acetate [a]

Tissue	v_0
Liver	27.10 ± 4.20
Kidneys	10.57 ± 3.72
Brain	3.02 ± 0.22

[a] Results are averages of three determinations, expressed in nanomoles of II per minute per milligram of protein, at pH 7.5 and 37° in saline.

0.32 *M* sucrose. The protein content of the fractions was determined by the biuret reaction (12).

Esterase Activity—The esterase activity of the tissue homogenates and their subcellular fractions was determined titrimetrically by pH-stat[5] in 20 ml of 0.9% NaCl containing 1–50 mg of protein. The initial concentration of β-naphthyl acetate was 1 m*M*, and that of II was 0.5 m*M*. The initial hydrolysis rate was determined at 37°, and the pH was maintained at 7.5 by addition of 0.01 *N* NaOH.

Preparative Hydrolysis of II—*In vitro* hydrolysis was stopped at 50% conversion, and the remaining ester was extracted by ether and purified on a preparative thin-layer sheet. NMR spectra were obtained[6]. A europium optishift reagent[7] was used for the determination of the ratio of the isomers. Circular dichroism spectra were taken[8] in methanol. Molecular ellipticities (θ_{223}) were calculated at 223 nm.

RESULTS AND DISCUSSION

***In Vivo* Hydrolysis**—The relative tissue binding of I and II in a methanolic homogenate determined by an independent *in vitro* experiment was unity. Therefore, the ratio of radioactivities of the chromatographic spots corresponded to the ratio of the compounds in the tissues. During 30 min, only I and II contributed measurably to the total radioactivity.

Compound II, administered intravenously, hydrolyzed rapidly. Figure 1 shows the logarithm of this ratio as a function of time in brain and blood. At the first experimental point (1 min), the ratio of the concentrations of I and II was about unity. This result suggests an immeasurably rapid, stereospecific hydrolysis of the preferred isomer while the ratio decreases rapidly to 1 (log 1 = 0). Rapid hydrolysis seems to be the reason why II administered orally or intraperitoneally has pharmacological activity similar to I (1); *i.e.*, the ester itself can hardly reach its target organ. Thus, II is not a proper prodrug. However, it is a good model compound for *in vitro* examination of the esterases responsible for the bioactivation of ester-type prodrugs of I.

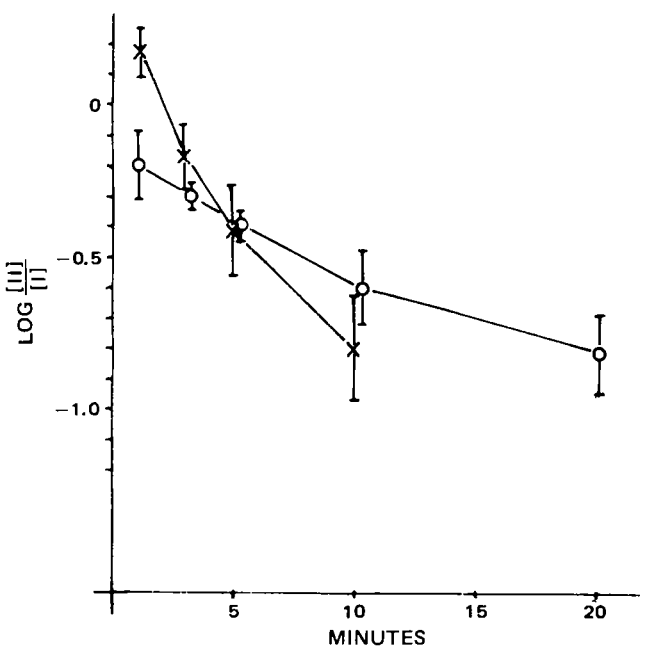

Figure 1—*Ratio of the concentrations of oxazepam acetate to oxazepam in blood (+) and brain (○). Each point represents five animals.*

[5] Radiometer, Copenhagen, Denmark.
[6] Varian XL-100.
[7] Tris[3-(heptafluoropropylhydroxymethylene)-*d*-camphorato]europium (III), Willow-Brook Co.
[8] Jouan-Quetin Dichrographe III.

Table II—Esterase Activity [a] of Liver and Brain Subcellular Fractions

Fraction	Liver		Brain	
	β-Naphthyl Acetate	Oxazepam Acetate	β-Naphthyl Acetate	Oxazepam Acetate
Total homogenate	1143 ± 11	27.1 ± 4.2	74 ± 3	3.02 ± 0.22
Nuclei plus cell debris	349 ± 24	32.9 ± 0.4	50 ± 2	1.88 ± 0.18
Mitochondrial	428 ± 60	46.7 ± 2.3	39 ± 4	2.05 ± 0.15
Microsomal	1810 ± 200	261.7 ± 5.0	125 ± 7	4.71 ± 0.49
Supernate	145 ± 6	2.1 ± 0.3	59 ± 2	5.37 ± 0.40

[a] Results are averages of three determinations in nanomoles of ester per minute per milligram of protein.

***In Vitro* Hydrolysis**—Table I lists the esterase activity of tissue homogenates. Esterase activity of the lungs and blood was not detectable by pH-stat, which does not mean, however, that they had no activity. For example, the hydrolysis of radiolabeled II in serum was detected following chromatographic separation of I. The major part of II decomposed in the liver and kidneys, but esterases also contributed to the process elsewhere. This was especially true for the brain, because its esterases may have modified the effective concentration of the drug at the site of action.

For the detection of stereoselectivity, the whole course of hydrolysis of racemic II was followed by a pH-stat. Titration curves show double exponential character, with a breakpoint at half-conversion of the ester. This phenomenon was significant only for liver and brain homogenates.

Thus, both *in vivo* and *in vitro* kinetic data support the assumption that the esterases in question are stereospecific. Chemical composition and chirality of II isolated by preparative hydrolysis also verify this assumption. The NMR spectrum was identical with the reference substance. On addition of the europium optishift reagent, the proton attached to C-3 (originally at 6.0 ppm) gave two signals of different intensity. With the liver homogenate, the ratio of the isomers was 20:80. The polarimetric rotation results also indicate differing stereospecificities. The $[\alpha]_D$ value of II extracted from liver homogenate was 90°, and that of II extracted from brain homogenate was −56° (c 0.5, dioxane).

Circular dichroism spectra (Fig. 2) show that esterases in liver and brain homogenates had opposite stereospecificity. The spectra were similar to those of oxazepam succinate half-ester (III) (13, 14), and the absolute configuration of the isomers can be established. This identification cannot be approached from the I side because it racemizes rapidly in an aqueous medium (13).

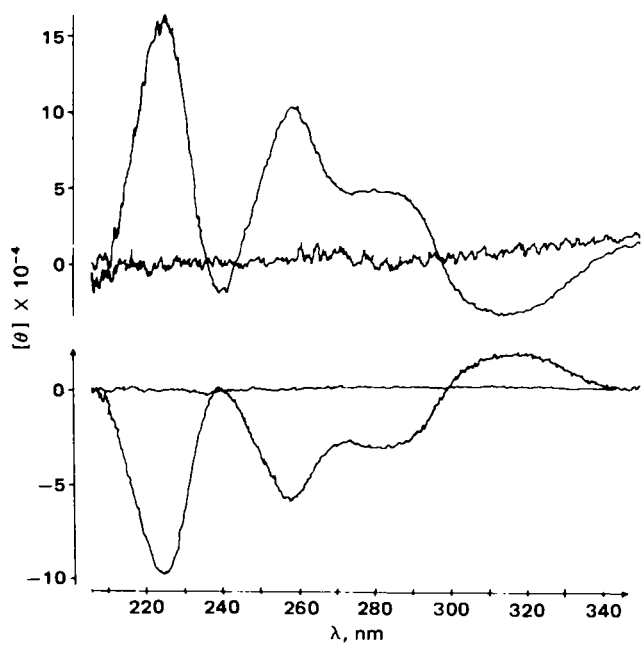

Figure 2—*Circular dichroism spectra of oxazepam acetate partially hydrolyzed by total liver [upper spectrum, (S)-(+)-isomer] and brain [lower spectrum, (R)-(−)-isomer] homogenates.*

Table III—Optical Activity of Partially Hydrolyzed Oxazepam Acetate

Fraction	θ_{223} Liver	θ_{223} Brain
Mitochondrial	19,800	−125,400
Microsomal	42,900	2,310
Soluble	330	−16,500

Stereospecificity was examined in liver and brain homogenates of different species. Indexes of absolute configuration based on the sign of polarimetric rotation of partially hydrolyzed II were (*S*)-(+) in all liver homogenates and (*R*)-(−) in all brain homogenates from mice, rats, chickens, pigs, and cows. These data show that the difference was general for all species tested.

Subcellular Localization of Esterases in Brain and Liver of Mice—The enzymatic activity of the subcellular fractions for oxazepam acetate was determined in a nearly saturated solution of the substrate. Esterase activity was also measured with a nonspecific substrate, β-naphthyl acetate (Table II).

Microsomal proteins had a high specific activity, in agreement with previous findings for the liver (15, 16) and brain (17). Salmona *et al.* (6) reported that III was hydrolyzed only by the 105,000×*g* supernate of mouse liver and kidney homogenate and that the hydrolysis rate of the isomers was different. Compound II hydrolyzed more readily than III, and its stereospecificity was opposite to that of III. Probably this difference was due to different enzymes such as acetic esterases.

For the localization of the different stereoselectivity of brain and liver tissues, the optical activity of II partially hydrolyzed by tissue fractions was measured. Because the 900×*g* pellets formed a rather heterogeneous fraction containing nuclei and cell debris (17), they were excluded from the investigation. Table III lists the molecular ellipticities (θ_{223}) of II.

To estimate the contribution of the fractions to the total stereoselectivity, their participation in the total esterase activity must be considered. The data in Table IV show the total esterase activity of the subcellular fractions calculated for 1 g of wet tissue.

Liver—Microsomal esterases had a decisive role both in activity and selectivity. The soluble fraction had almost no stereoselectivity for racemic II, in contrast to the results of Salmona *et al.* (6) who found this fraction to be selective for the resolved isomers of III.

Brain—Because of its high yield, the crude mitochondrial fraction overcompensated the effect of the microsomal fraction and determined the opposite stereospecificity of brain tissue. De Robertis *et al.* (11) showed that the crude mitochondrial fraction of rat brain contained synaptosomes. Bernsohn *et al.* (17) found that esterase activity of this fraction was related to synaptic membranes. In this case, esterases of the supernate with similar stereospecificity might be of the same origin. At any rate, the phenomenon is not connected with cholinesterases, because physostigmine at up to 2×10^{-3} *M* concentration neither inhibited nor modified stereospecificity.

The opposite stereoselectivity of different organs is an interesting phenomenon. It is similar to earlier findings (18) for the hydrolysis of methyl mandelate by pig liver and pancreas esterases. Peculiarly, however, the stereoselectivity of pig liver was different toward the racemate and the resolved enantiomers (19). To investigate such a possibility, resolution of the isomers of II is in progress. The question arises whether the phenomenon of opposite stereoselectivity exists for other asymmetric esters, which would be significant in the biotransformation of several centrally acting drugs.

Table IV—Total Esterase Activity [a] of the Subcellular Fractions

Fraction	Liver	Brain
Total homogenate	18,420	606.7
Nuclei plus cell debris	1,220	126.0
Mitochondrial	3,060	193.3
Microsomal	9,890	95.1
Soluble	200	126.7

[a] Values are in nanomoles of II per minute per gram of tissue.

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