In Vitro Hydrolysis of Oxazepam Succinate Half-Ester by a Stereospecific Soluble Esterase from Different Animal Species

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Abstract □ The presence of a stereospecific soluble esterase that hydrolyzes the three forms of oxazepam succinate half-ester [(+), (−), and (±)] in some tissues of rats, mice, and guinea pigs was demonstrated. The enzymatic activity was determined by a titrimetric method. The K_m values obtained were 2.5×10^{-3} *M* for the (+)-isomer, 3.3×10^{-3} *M* for the racemate, and 14.3×10^{-3} *M* for the (−)-isomer in tissues showing enzymatic activity. Studies employing several inhibitors or activators indicate that the enzyme probably belongs to the C-esterase group.

Keyphrases □ Oxazepam succinate half-ester—*in vitro* hydrolysis by stereospecific soluble esterase from rats, mice, and guinea pigs □ Stereospecific esterase in liver, kidney, and brain of rats, mice, and guinea pigs—*in vitro* hydrolysis of oxazepam succinate half-ester □ Enzymatic activity, stereospecific soluble esterase in rat, mouse, and guinea pig—*in vitro* hydrolysis of oxazepam succinate half-ester □ Esterase, stereospecific, in rats, mice, and guinea pigs—*in vitro* hydrolysis of oxazepam succinate half-ester

A recent derivative of oxazepam, the succinate half-ester which differs from the parent compound due to its high solubility in water, shows interesting pharmacodynamic and kinetic properties (1–4). The racemic compound has been resolved into the two optically active isomers (5). These enantiomers show quantitatively different pharmacological effects. Mussini *et al.* (6) recently demonstrated that the (+)-isomer shows a more powerful antipentylenetetrazol effect than the (−)-isomer while the racemate exhibits intermediate activity. More recently, de Angelis *et al.* (7), studying the effects of oxazepam succinate half-ester in the central nervous system (CNS), also showed that the (+)-isomer is more active than the (−)-form while the activity of the racemate lies between the two. The tests used to evaluate their activity in mice were potentiation of narcosis, spontaneous motor activity, antipentylenetetrazol and antistrychnine activities, analgesic effect, and muscle relaxant activity.

Since the pharmacological activity of oxazepam succinate half-ester may depend on the availability of oxazepam (6), an attempt was made to localize the distribution of the esterase that metabolizes this compound in three animal species and to confirm its stereospecificity *in vitro*. Furthermore, various inhibitors were used to investigate the type of esterase involved in the hydrolysis of the ester linkage between oxazepam and succinate.

EXPERIMENTAL

Animals—Male Sprague–Dawley rats (200–250 g), male albino Swiss mice (20–25 g), and male albino guinea pigs (300–350 g), fed *ad libitum*, were used in all experiments.

Substrates—α-Naphthyl acetate was synthesized from acetic anhydride and α-naphthol[1] by shaking an ice-cold aqueous alkaline solution of α-naphthol with acetic anhydride. The acetyl derivative, crystallized from water had a melting point of 48.5°.

The following substrates were also used: oxazepam succinate half-esters[2], neostigmine methylsulfate[1], physostigmine[1] (eserine), diisopropylfluorophosphate[1] (I), edetate[1] (ethylene diaminetetraacetate) (II), *p*-chloromercuribenzoate[3] (III), mercuric chloride[4], sodium fluoride[5], and lanthanum nitrate hexahydrate[5] [$La(NO_3)_3 \cdot 6H_2O$].

Buffers—For the enzymatic determinations, either naphthyl acetate or the oxazepam succinate half-ester was dissolved in 0.025 *M* phosphate buffer, pH 7.6. For the experiments concerning the pH-activity profile, the following buffers were used: 0.025 *M* sodium acetate for pH 5.0–6.5, 0.025 *M* sodium phosphate for pH 6.5–8, 0.025 *M* sodium pyrophosphate for pH 8–9.5, and 0.025 *M* sodium borate for pH values above 9.5. The pH of all solutions was adjusted to the desired value before the experiments.

Preparation of Microsomes—After killing the animals, the liver, kidneys, heart, lungs, and brain were immediately removed and homogenized in ice-cold 1.15% KCl solution (1:4 w/v) with a Teflon glass homogenizer.

The homogenate was centrifuged at 9000×*g* for 20 min, and the supernatant fraction was further centrifuged[6] at 105,000×*g* for 1 hr. The supernatant fractions containing the "soluble" enzymes were collected separately, and the microsomes were suspended in 1.15% KCl solution.

Blood Collection—Animals were killed by decapitation, and blood samples were collected in heparinized test tubes. After centrifugation at 9000×*g* for 10 min, the erythrocytes and plasma were collected separately and tested for enzymatic activities.

Protein Analysis—Proteins were determined according to the method of Lowry *et al.* (8).

Enzymatic Activities—The esterase activity of the aqueous extracts was determined by a titrimetric method[7] (9). The volume of the reaction mixture was always 15 ml. A volume of 0.100–0.300 ml or a suitable dilution of enzymatic preparation was added to the reaction vessel. The final concentration of α-naphthyl acetate or of oxazepam succinate half-ester in the vessel was 10^{-3} *M*. The reaction mixture also contained 0.45% NaCl.

Sodium hydroxide at a concentration of 0.005 *M* was used for the titrations, which were carried out at 37° for 3 min, maintaining the pH at 7.6. The initial rate of hydrolysis was determined from the titration curves as micromoles of sodium hydroxide utilized per milliliter of extract per minute.

Studies with Inhibitors (or Activators)—To investigate the effect of a number of inhibitors (or activators) of esterases on the enzyme metabolizing the oxazepam succinate half-ester, the normal reaction mixture was added together with 1 ml or a suitable dilution of inhibitor (or activator) to obtain a final concentration range of 10^{-8}–10^{-3} *M*. The titration was then carried out as usual.

The I_{50} values, *i.e.*, the concentration of inhibitors producing 50% of inhibition, were obtained graphically by plotting the per-

[1] Merck, Darmstadt, Germany.
[2] RV 1206 (±), RV 1208 (+), and RV 1210 (−), supplied by U. Ravizza, Muggiò, Milan, Italy.
[3] Shuchardt, München, Germany.
[4] Industria Chimica Italo-Svizzera, Milan, Italy.
[5] Carlo Erba, Milan, Italy.
[6] Beckman model L ultracentrifuge, rotor 40′.
[7] pH-Stat TTT Ic apparatus, Radiometer, Copenhagen, Denmark.

Table I—Esterase Activity in Rats (Units per Milligram Protein)[a]

Tissue	α-Naphthyl Acetate	Oxazepam Succinate Half-Ester (±)	(+)	(−)
Plasma	0.146 ± 0.010	—	—	—
Brain				
Soluble fraction	0.285 ± 0.020	—	—	—
Microsomes	0.773 ± 0.030	—	—	—
Liver				
Soluble fraction	0.032 ± 0.040	0.021 ± 0.003	0.053 ± 0.003	0.017 ± 0.003
Microsomes	1.043 ± 0.072	—	—	—
Kidney				
Soluble fraction	0.962 ± 0.270	—	—	—
Microsomes	5.340 ± 0.180	—	—	—

[a] Results are expressed as mean ± standard error of six determinations. A pool of three rats was used for each experiment.

Table II—Esterase Activity in Mice (Units per Milligram Protein)[a]

Tissue	α-Naphthyl Acetate	Oxazepam Succinate Half-Ester (±)	(+)	(−)
Plasma	0.121 ± 0.012	—	—	—
Brain				
Soluble fraction	0.485 ± 0.070	—	—	—
Microsomes	3.680 ± 0.470	—	—	—
Liver				
Soluble fraction	0.427 ± 0.023	0.015 ± 0.001	0.040 ± 0.002	0.004 ± 0.001
Microsomes	0.868 ± 0.074	—	—	—
Kidney				
Soluble fraction	1.925 ± 0.020	1.111 ± 0.030	1.501 ± 0.025	0.925 ± 0.014
Microsomes	1.820 ± 0.045	—	—	—

[a] Results are expressed as mean ± standard error of six determinations. A pool of 10 mice was used for each experiment.

Table III—Esterase Activity in Guinea Pigs (Units per Milligram Protein)[a]

Tissue	α-Naphthyl Acetate	Oxazepam Succinate Half-Ester (±)	(+)	(−)
Plasma	0.407 ± 0.031	—	—	—
Brain				
Soluble fraction	0.056 ± 0.001	0.023 ± 0.001	0.006 ± 0.001	0.019 ± 0.001
Microsomes	0.202 ± 0.054	—	—	—
Liver				
Soluble fraction	0.490 ± 0.010	0.143 ± 0.010	0.380 ± 0.050	0.113 ± 0.020
Microsomes	6.640 ± 0.298	—	—	—
Kidney				
Soluble fraction	0.605 ± 0.001	0.025 ± 0.003	0.031 ± 0.001	0.018 ± 0.001
Microsomes	1.550 ± 0.328	—	—	—

[a] Results are expressed as mean ± standard error of six determinations. A pool of three guinea pigs was used for each experiment.

centage of inhibition as a junction of inhibitor concentration on semilogarithmic paper.

RESULTS

Tissue Distribution of Enzyme—To find the exact distribution of the enzyme metabolizing the oxazepam succinate half-ester, the total amount of esterase activity present in the extracts was initially determined by means of α-naphthyl acetate, since it can be metabolized, although at a different rate, by all esterases (10–12). Then the extracts were analyzed for oxazepam succinate half-ester hydrolyzing activity. Tables I–III show the tissue distribution of the enzyme in rats, mice, and guinea pigs, respectively.

Esterase activity was not detected with respect to the oxazepam succinate half-ester in the heart, lungs, and erythrocytes of these three animal species. In contrast to oxazepam succinate, enzyme activity against α-naphthyl acetate was found in both supernatant fractions and in the microsomes of the tissues studied.

The distribution of oxazepam succinate esterases was not the same in the three animal species; when the enzyme was present, it was only in the soluble fractions of the cells, *i.e.*, in the supernatant fraction of the 105,000×*g* preparation. Although the enzyme always showed a stereospecific activity on oxazepam succinate half-ester, only in the soluble fraction of liver does this difference seem to be important.

K_m Values for (+)- and (−)-Isomers and for the Racemate in Tested Tissues—The K_m values obtained were 2.5×10^{-3} *M* for the (+)-isomer, 3.3×10^{-3} *M* for the racemate, and 14.3×10^{-3} *M* for the (−)-isomer. The K_m values for the three forms of oxazepam succinate half-ester did not differ significantly among the three species studied (Fig. 1).

Studies with Inhibitors (or Activators) of Esterases—The enzymatic preparations were first tested with neostigmine, physostigmine, and I. These compounds did not show any inhibitory activity toward the enzyme. However, I was not hydrolyzed by the enzymatic preparations. Cupric sulfate and mercuric chloride inhibited the enzymatic preparations very strongly (Fig. 2); the pI_{50} values for the two salts were 5 and 6, respectively. Compound III activated the enzyme when used at low concentrations in the 10^{-6}–10^{-4} *M* range, but at 10^{-3} *M* this activation dimin-

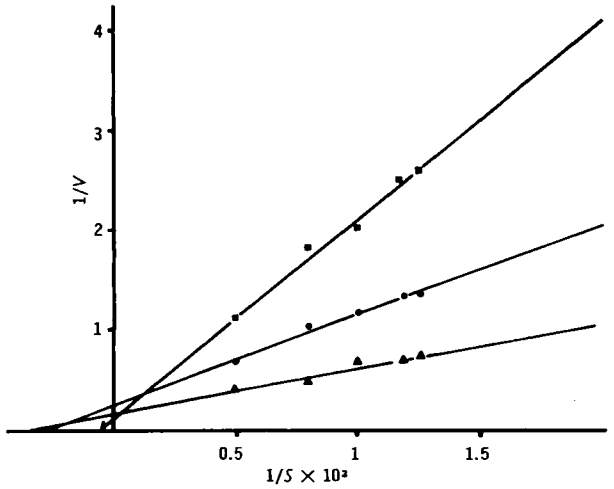

Figure 1—*Double reciprocal plot of the three forms of oxazepam succinate half-ester. In all tissues with oxazepam succinate esterase activity, the* K_m *values do not differ significantly in the three animal species studied. The* K_m *values are* 3.3×10^{-3} M *for the racemate* (●), 2.5×10^{-3} M *for the* (+)*-isomer* (▲), *and* 14.3×10^{-3} M *for the* (−)*-isomer* (■). V *is the initial velocity of the reaction in micromoles per minute per milligram protein.* S = [*oxazepam succinate half-ester* (M)].

ished (Fig. 3). The maximum activation occurred at a concentration of 10^{-5} *M*. Compound II and sodium fluoride (Fig. 3) also activated the enzymatic preparations at low concentrations, although this effect was not comparable with that of III.

pH-Activity Profile for Oxazepam Succinate Half-Ester Esterases—The pH-activity profile for oxazepam succinate half-ester of enzymatic preparations is bell-shaped and has a "plateau" between pH 7.5 and 8 (Fig. 4).

DISCUSSION

The enzyme that is able to metabolize the succinate half-ester of oxazepam is located only in the supernatant fraction of the 105,000×*g* preparations, which means that the enzyme is not particulate but belongs to the soluble enzymes of the cell. The different distribution of enzymes in the three species studied may be due to a different phylogenetical development or to a peculiar difference of enzymatic content in these three species. In man, for instance, it is well known that many genetic differences in esterasic response can be found (13–17) for different substrates.

The behavior of the enzymatic preparations against the inhibitors or activators studied seems very similar to that found in previous studies (18–22). In fact, the enzymatic preparations are not inhibited by organophosphates while they are activated by III at low concentrations; at higher concentrations, a degradation of enzymatic protein probably occurs. It must be emphasized that while Bergmann and Rimon (21) reported a maximum of 800% activation, only 400% activation was found in the present preparations. On the other hand, Tashian (20) reported an activation at high concentrations, *i.e.*, 10^{-3} *M*. Furthermore, sodium fluoride and II at low concentrations activate the enzymatic preparations. The II-dependent activity is quite unusual, since Björklund *et al.* (23) reported a II-dependent A-esterase in human blood.

Although there is no evidence in the literature of enzymatic activation by sodium fluoride, since this compound normally inhibits pseudocholinesterases (24, 25), the present findings may be interesting with regard to the further characterization of the enzymes, probably belonging to the C-esterase group. The lanthanum salt does not inhibit the enzymatic preparations even at a concentration of 10^{-3} *M*. Mercuric chloride and cupric sulfate strongly inhibit the enzymatic activity at very low concentrations; in the experimental conditions, the pI_{50} values were 5 and 6, respectively. The type of inhibition involved was not identified since this will be done after purification of the enzyme to eliminate any possible interaction.

The results obtained suggest that the hydrolysis of oxazepam succinate half-ester may show specific aspects *in vivo* in the three animal species tested.

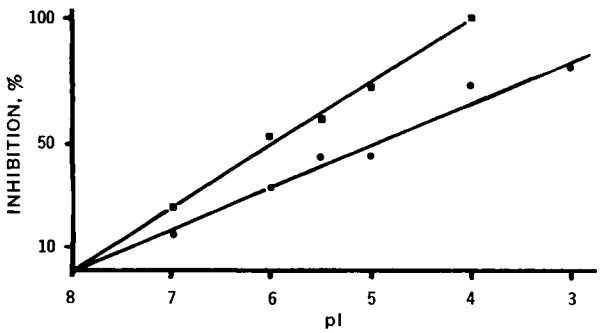

Figure 2—*The pI–activity curve of the enzymatic preparations. Key:* ●, *mercuric chloride; and* ■, *cupric sulfate. pI = −log of concentration of inhibitor.*

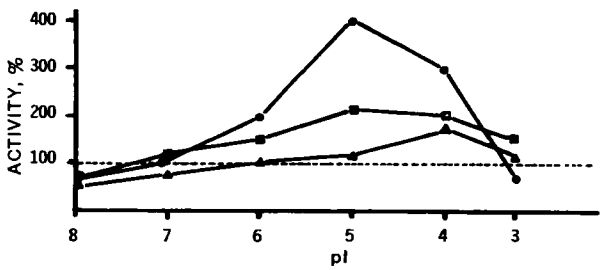

Figure 3—*Effect of* p-*chloromercuribenzoate* (●), *edetate* (▲), *and sodium fluoride* (■) *on the enzymatic preparations. The horizontal line indicates the standard level of activity.*

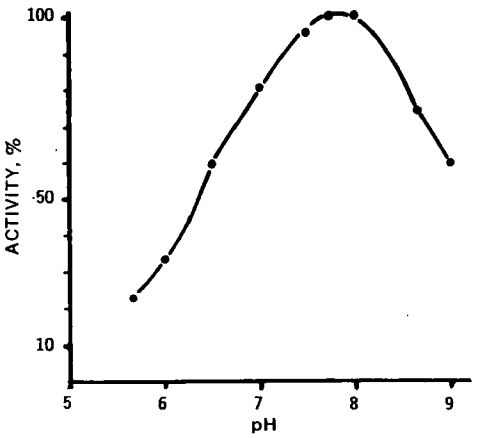

Figure 4—*The pH–activity profile of enzymatic preparations, showing a "plateau" between pH 7.5 and 8.*

Table IV—Behavior of Enzymatic Preparations Hydrolyzing Oxazepam Succinate Half-Ester against Inhibitors (or Activators)

Enzymatic Preparations	Activity
Neostigmine	No inhibition
Physostigmine	No inhibition
Diisopropylfluorophosphate	No inhibition, no esterase activity for this compound
p-Chloromercuribenzoate	Activation at 10^{-5} *M*
Edetate	Activation at 10^{-4} *M*
Sodium fluoride	Activation at 10^{-5} *M*
Mercuric chloride	Inhibition, $pI_{50} = 5$
Cupric sulfate	Inhibition, $pI_{50} = 6$
Lanthanum nitrate hexahydrate	No inhibition

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Disposition of Hydralazine in Man and a Specific Method for Its Determination in Biological Fluids

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Abstract □ A sensitive analytical procedure was developed for the measurement of the concentrations of hydralazine in human plasma and urine. The method is based on the formation of the hydrazone derivative of hydralazine with *p*-methoxybenzaldehyde. Specificity of the procedure for unchanged drug was demonstrated by inverse isotope dilution analysis following administration of hydralazine-^{14}C to humans. Plasma levels and urinary excretion of hydralazine were measured in healthy subjects after single oral therapeutic doses.

Keyphrases □ Hydralazine-^{14}C—plasma levels and urinary excretion in man, spectrophotometric and inverse isotope dilution analysis in biological fluids □ Plasma levels—hydralazine-^{14}C after oral administration, man □ Urinary excretion—hydralazine-^{14}C after oral administration, man □ Spectrophotometry—analysis, hydralazine in plasma and urine □ Inverse isotope dilution—analysis, hydralazine in plasma and urine

Hydralazine[1] (1-hydrazinophthalazine), a potent, peripherally acting vasodilator with a prompt and general blood pressure-lowering effect, has been used for the treatment of essential hypertension for many years. Although many pharmacological and clinical papers have appeared, relatively little is known concerning its biological disposition.

The fate of hydralazine in the rat was investigated (1) with the aid of ^{14}C-labeled material. Plasma levels of hydralazine in man after oral administration were reported (2, 3), and average peak levels of 0.23 μg/ml were attained after a daily dose of 600 mg. In another study (4), 150 mg of hydralazine was given four times daily for an extended period to one hypertensive subject and peak plasma levels reached a plateau of 1.5 μg/ml. Recently, plasma levels 2 hr postadministration obtained in hypertensive patients after 5–20 weeks of hydralazine treatment were reported (5).

The objective of the present study was to obtain information on the biological fate of hydralazine in healthy human subjects after administration of single doses in the clinically effective dose range. Based on the reported data, it was anticipated that plasma levels after administration of a single 100-mg dose would decline rapidly. Therefore, the sensitivity and specificity of existing assay methods had to be examined carefully.

The method published by Perry *et al.* (3) utilized

[1] Apresoline, Ciba-Geigy Corp.