Biochem. J. (1990) 268, 409–414 (Printed in Great Britain)

DL-Canaline and 5-fluoromethylornithine

Comparison of two inactivators of ornithine aminotransferase

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5-Fluoromethylornithine (5FMOrn) is an enzyme-activated irreversible inhibitor of ornithine aminotransferase (L-ornithine:2-oxo-acid 5-aminotransferase, OAT). For purified rat liver OAT, $K_{i(app.)}$ was found to be 30 μM. and $\tau_{\frac{1}{2}} = 4$ min. Of the four stereomers of 5FMOrn only one reacts with OAT. The formation of a chromophore with an absorption maximum at 458 nm after inactivation of OAT by 5FMOrn suggests the formation of an enamine intermediate, which is slowly hydrolysed to release an unsaturated ketone. L-Canaline [(*S*)-2-amino-4-amino-oxybutyric acid] is a well-known irreversible inhibitor of OAT. Not only the natural L-enantiomer but also the D-enantiomer reacts by oxime formation with pyridoxal 5'-phosphate in the active site of the enzyme, although considerably more slowly. This demonstrates that the stereochemistry at C-2 of ornithine is not absolutely stringent. *In vitro*, canaline reacted faster than 5FMOrn with OAT. *In vivo*, however, only incomplete OAT inhibition was observed with canaline. Whereas intraperitoneal administration of 10 mg of 5FMOrn/kg body wt. to mice was sufficient to inactivate OAT in brain and liver by 90% for 24 h, 500 mg of DL-canaline/kg body wt. only produced a transient inhibition of 65–70%. The accumulation of ornithine in these tissues was considerably slower and the maximum concentrations lower than were achieved with 5FMOrn. It appears that DL-canaline, in contrast with 5FMOrn, is not useful as a tool in studies of biological consequences of OAT inhibition.

INTRODUCTION

L-Canaline [(*S*)-2-amino-4-amino-oxybutyric acid] (Rosenthal, 1978) and 5-fluoromethylornithine (6-fluoro-2,5-diaminohexanoic acid, 5FMOrn) (Daune *et al.*, 1988) have been shown to inhibit ornithine aminotransferase (L-ornithine:2-oxo-acid 5-aminotransferase, OAT, EC 2.6.1.13) irreversibly. In the case of canaline mechanistic aspects of OAT inhibition have previously been reported only for the natural L-isomer (Sanada *et al.*, 1976; Kito *et al.*, 1978). *In vivo*, studies with canaline have been restricted to intracerebroventricular injections of microgram quantities, as far as vertebrates are concerned (Wroblewski *et al.*, 1985; McCown *et al.*, 1987). Effects of systemic canaline administration seem not to have been reported. The situation concerning 5FMOrn is opposite to that of canaline: 5FMOrn is a mixture of two diastereomers, each consisting of a pair of enantiomers. The properties of the individual stereomers are not known. However, the effects of single and multiple injections of 5FMOrn on ornithine metabolism have been extensively studied (Daune, 1988; Daune *et al.*, 1988; Seiler *et al.*, 1989).

In order to fill in a gap in our knowledge of the two most potent inhibitors of OAT, a comparison of canaline and 5FMOrn seemed appropriate.

MATERIALS AND METHODS

Chemicals

If not stated otherwise, chemicals and solvents were from Aldrich-Chimie (Strasbourg, France), Merck (Darmstadt, Germany) or Baker Chemicals (Deventer, The Netherlands). 4-Fluoromethylornithine hydrochloride (5FMOrn, MDL 72912) was prepared as described previously (Daune *et al.*, 1988). DL-Canaline was synthesized by the method of Knobler & Frankel (1958).

L-Canaline and *N*-acetyl-L-cysteine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and (+)-1-(9-fluorenyl)ethyl chloroformate was from Interchim (Montluçon, France).

Laboratory animals

CD1 albino mice (male, weighing 35±5 g) and Sprague–Dawley rats (male, weighing 200–300 g) were from Charles River (St.-Aubin-les-Elbeuf, France). They were kept at standardized conditions (22 °C; 60% relative humidity; 12 h-light–12 h-dark cycle; water and standard diet *ad libitum*).

Purification of OAT from rat liver

This was performed by using the method of Peraino *et al.* (1969) with the modifications introduced by Williams *et al.* (1982). The specific activity of the final preparation was 1 mmol of Δ^1-pyrroline-5-carboxylic acid formed/h per mg of protein. Separation by SDS/PAGE followed by staining with $AgNO_3$ showed one major band at 45 kDa and an impurity (< 1%) at 91 kDa.

Determination of OAT activity

In enzyme assays *in vitro* OAT activity was determined spectrophotometrically by the method of Strecker (1965), except that higher substrate concentrations (175 mM-L-ornithine and 35 mM-2-oxoglutarate) were used. For the sensitive determination of OAT in tissue homogenates a chromatographic version of the method of Strecker (Seiler *et al.*, 1987) was used.

Determination of ornithine

Ornithine was assayed in $HClO_4$ tissue extracts by derivative formation with *o*-phthalaldehyde/2-mercaptoethanol, separation of the resulting bis-isoindole derivative from the derivatives of other amino acids and detection by using an electrochemical detector (Seiler *et al.*, 1987).

Abbreviations used: OAT, ornithine aminotransferase; 5FMOrn, 5-fluoromethylornithine.

Determination of the active enantiomer of 5FMOrn

In a total volume of 400 μl, OAT (0.4 nmol) was incubated with 1.6 nmol or 4 nmol of 5FMOrn under the conditions that were used for the assay of time-dependent incubation. After different times of incubation, 40 μl portions were added to 40 μl of 0.2 M-$HClO_4$, and 50 μl of each of these mixtures was allowed to react for exactly 5 min with 50 μl of the *o*-phthalaldehyde/*N*-acetyl-L-cysteine reagent (Buck & Krummen, 1987). The four diastereomeric bis-(2-alkyl-1-thioalkyl-isoindoles) formed from 5FMOrn were separated in 20 μl portions of the reaction mixture on a reversed-phase h.p.l.c. column (Beckmann Ultrasphere I.P.). Separation was achieved by isocratic elution (flow rate 1 ml/min) with 45% (v/v) methanol in 0.2 M-sodium acetate buffer, pH 4.5, as eluent. The isoindoles were monitored with an electrochemical detector (Coulochem model 5100 A; Environmental Sciences Associates, Bedford, MA, U.S.A.) with a dual analytical cell (model 5011). The coulometric electrode was used as a screen electrode at +0.05 V and the amperometric cell as detector electrode at +0.5 V (working potentials relative to the reference electrode). The current resulting from the oxidation of the bis-isoindoles was recorded as a function of elution time. The reagent was prepared as follows. Sodium borate buffer was prepared by dissolving 5.0 g of H_3BO_3 and 4.4 g of KOH per 100 ml of water. *o*-Phthalaldehyde (54 mg) was dissolved in 2 ml of methanol. This solution was mixed with 10 ml of sodium borate buffer and 244 mg of *N*-acetyl-L-cysteine. (This reagent is analogous to that used for ornithine determination, except that 2-mercaptoethanol was replaced by an equivalent amount of *N*-acetyl-L-cysteine.) The method is also suitable for the separation of the ornithine enantiomers.

Separation and determination of the enantiomers of canaline

DL-Canaline was treated with (+)-1-(fluoren-9-yl)ethyl chloroformate (Einarsson *et al.*, 1987) as follows. A 20 μl sample solution in 0.2 M-$HClO_4$ was mixed with 20 μl of borate buffer (25 g of H_3BO_3 and 22 g of KOH per litre). To this solution was added 40 μl of (+)-1-(fluoren-9-yl)ethyl chloroformate solution (0.5 mg per 10 ml of acetone), and the reaction was allowed to proceed at room temperature for 10 min. Then 50 μl of 0.2 M-sodium acetate buffer, pH 4.5, was added, in order to neutralize the reaction mixture.

Under these conditions (+)-1-(fluoren-9-yl)ethyl chloroformate reacts nearly exclusively with the α-amino group of canaline. In the range from 0.5 to 2 nmol/ml the yield of (+)-1-(fluoren-9-yl)ethyl chloroform amide was directly proportional to the amount of DL-or L-canaline in the sample solution.

For separation a 100 μl portion was applied on a reversed-phase h.p.l.c. column (250 mm length × 4.6 mm internal diam.) [Superspher RP-18 (3 μm particle size); Bischoff, Leonberg, Germany]. Elution was achieved at a flow rate of 1 ml/min (at 25 °C) by using the following gradient: eluent A in eluent B continuously decreasing from 60% (v/v) to 43% (v/v) within 20 min and eluent C from 21 min to the end time 35 min, with equilibration with the initial buffer composition for 8 min between each run. Eluent A was 10% (v/v) acetonitrile in 0.1 M-sodium acetate buffer, pH 4.50, eluent B was 70% (v/v) acetonitrile/5% (v/v) methanol in 0.1 M-sodium acetate buffer, pH 4.50, and eluent C was methanol (the purpose of the elution with methanol was to wash the column).

For the quantitative determination of the (+)-1-(fluoren-9-yl)ethyl chloroformate derivatives of the canaline enantiomers the fluorescence intensity of the column eluate was monitored, with excitation at 266 nm and emission at 310 nm. The (+)-1-(fluoren-9-yl)ethyl chloroformate derivative of D-canaline was eluted at 17.0±0.1 min and the (+)-1-(fluoren-9-yl)ethyl chloroformate derivative of L-canaline at 17.5±0.1 min.

C.d.

Before spectral determinations 1 mg of OAT was incubated in 0.6 ml of 50 mM-potassium phosphate buffer, pH 8.0, for 90 min at 37 °C in the absence or in the presence of either 5 μmol of 5FMOrn or 5 μmol of ornithine. Incubations were followed by 48 h dialysis at 4 °C against 500 ml of the phosphate buffer (two changes).

C.d. studies of the dialysed solutions were performed with an AVIV model 62 D spectropolarimeter, which had been calibrated by using (+)-camphor-10-sulphonic acid (Tuzimura *et al.*, 1977). Spectra of OAT at 49 μg per ml of phosphate buffer were recorded in cells with a pathlength of 5 mm at 37 °C; the bandwidth was 1.5 nm, measurements were in 0.5 nm steps and the time constant was 4 s. A total of five scans were averaged for both sample and solvent. After correction of the sample spectrum for solvent contributions, the data were fitted by non-linear regression analysis.

Instrumentation

For the h.p.l.c. separations a Varian model Vista 5500 liquid chromatograph was used in conjunction with a Hewlett–Packard model HP 1046A programmable fluorescence detector and a Milton–Roy CI 10-B integrator. The absorption spectra were obtained with a Beckman DU-7 spectrophotometer.

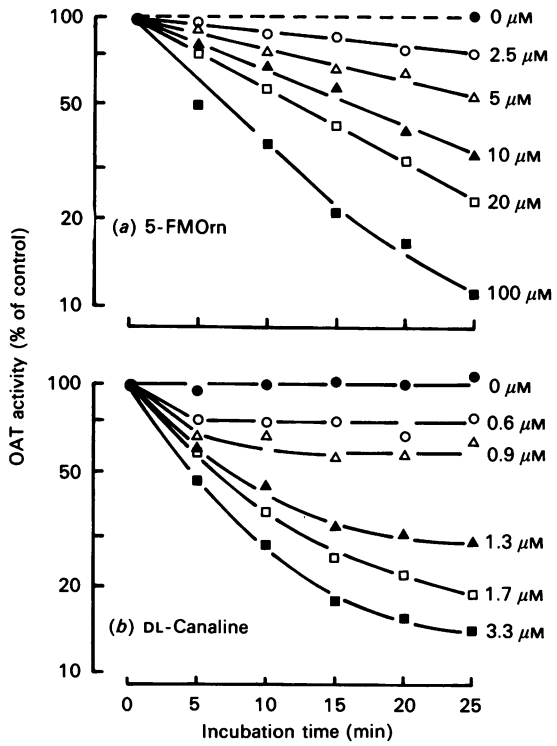


Fig. 1. Time-dependent inactivation of OAT by (*a*) 5-FMOrn and (*b*) DL-canaline

OAT (8 units; 1 unit represents 1 μmol of Δ^1-pyrroline-5-carboxylate formed/h) was incubated at 37 °C with the inhibitors in 50 mM-potassium phosphate buffer, pH 8.0 (total volume 150 μl). Samples (15 μl) were taken at fixed time intervals and diluted with 500 μl of potassium phosphate buffer containing 0.1 mM-pyridoxal 5'-phosphate, 175 mM-L-ornithine and 35 mM-2-oxoglutarate. The residual OAT activity was determined as described in the Materials and methods section. Because OAT activity was already inactivated to a certain proportion a few seconds after the addition of DL-canaline, the experimentally determined 'initial' activities were taken as 100% for the calculation of the rate of time-dependent inactivation.

Determination of proteins

For the determination of total proteins, the Lowry method, as modified by Hartree (1972), was used with BSA as standard. The amount of active OAT was determined as described by Williams *et al.* (1982) by using the decrease in pyridoxal absorbance at 412 nm following incubation of the enzyme preparation with 5 mM-L-ornithine.

Table 1. Effect of dialysis and incubation with substrates on the activity of inhibitor-pretreated OAT

OAT was first incubated with 500 μM-5FMOrn or 100 μM-DL-canaline for 60 min at 37 °C in 50 mM-potassium phosphate buffer, pH 8.0. The incubation mixtures (2 ml) were then dialysed for 20 h against 3 × 50 ml of 50 mM-potassium phosphate buffer, pH 8.0, containing 0.1 mM-pyridoxal 5′-phosphate, followed by incubation at 37 °C with substrates. The values are expressed as means ± S.D. ($n = 3$). Control activity (after preincubation, 20 h dialysis and 75 min incubation in the absence of substrates) was 621 ± 10 μmol/h per mg.

Treatment	Inhibitor...	OAT activity (μmol of Δ^1-pyrroline-5-carboxylate/h per mg of protein) 5FMOrn	DL-Canaline
75 min incubation in the absence of substrates		57 ± 10	155 ± 5
75 min incubation in the presence of 59 mM-2-oxoglutarate		47 ± 4	157 ± 6
75 min incubation in the presence of 292 mM-L-ornithine		22 ± 4	89 ± 8

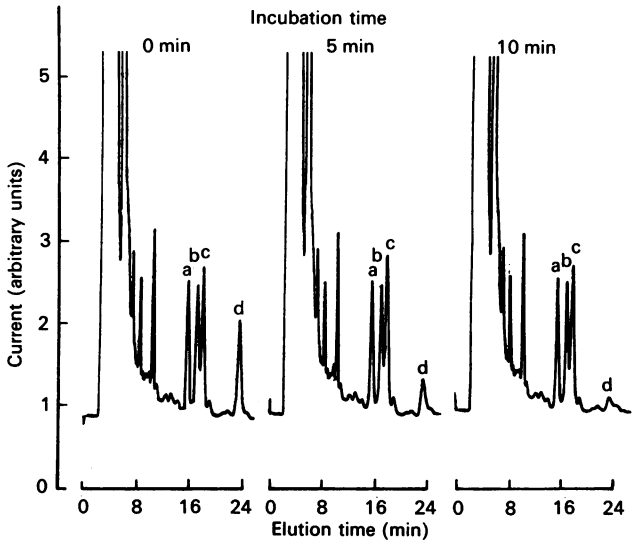


Fig. 2. Separation of the enantiomers of 5FMOrn after incubation with an equivalent amount of OAT

One equivalent of OAT was incubated with 4 equivalents of 5FMOrn. After certain time intervals the enzymic reaction was stopped with $HClO_4$. Portions of the $HClO_4$ solution were taken for reaction with *N*-acetyl-L-cysteine and *o*-phthalaldehyde, to form bis-(2-alkyl-1-thioalkylisoindoles). These were separated by h.p.l.c., and detected by means of an electrochemical detector, as described in detail in the Materials and methods section. Only one of the four stereomers disappeared as a result of incubation with OAT. This isomer (peak d) represents the active enantiomer of 5FMOrn.

RESULTS

Time-dependent inactivation of OAT by 5FMOrn and DL-canaline

Both 5FMOrn and DL-canaline inhibited OAT irreversibly in a time-dependent fashion (Fig. 1). The inactivation by 5FMOrn followed pseudo-first-order kinetics, but the reaction with DL-canaline did not follow simple kinetics. Plots of the enzyme half-lives (determined from initial reaction rates) against the reciprocal inhibitor concentration resulted in straight lines. These allowed us to determine the apparent dissociation constants ($K_{i(app.)}$) and the enzyme half-lives at infinite inhibitor concentration ($\tau_{\frac{1}{2}}$) by the method of Kitz & Wilson (1962). With 5FMOrn $K_{i(app)} = 30\ \mu M$ and $\tau_{\frac{1}{2}} = 4$ min, and with DL-canaline $K_{i(app)} = 2\ \mu M$ and $\tau_{\frac{1}{2}} = 2.5$ min. Neither extensive dialysis nor incubation with L-ornithine or 2-oxoglutarate (Table 1) re-activated the enzyme. This indicates that OAT inactivation by 5FMOrn and DL-canaline is indeed irreversible.

Stereospecificity of OAT inactivation by 5FMOrn

Only one of the four stereomers of 5FMOrn, namely peak d, disappeared (practically completely) from the reaction mixture upon incubation of inhibitor and OAT in a molar ratio of 4:1 (Fig. 2). It is therefore assumed that the inactivation reaction is stereospecific. From quantitative determinations of the disappearing peak d in incubations with excessive 5FMOrn, it was calculated that about 2 molecules of the active 5FMOrn stereomer were necessary to inactivate 1 molecule of OAT.

Inhibition of OAT by DL-canaline *in vitro*

If used in excess, L- and DL-canaline seemed to be equally effective in inhibiting OAT (Table 2). Preincubation with L-ornithine prevented the irreversible inhibition by canaline. It is known that the pyridoxamine-containing OAT, which is formed in the course of L-ornithine deamination, does not react with canaline. By adding 2-oxoglutarate to the incubation mixture pyridoxal 5′-phosphate in the active centre of OAT is regenerated from pyridoxamine (Sanada *et al.*, 1976) and thus inhibition by canaline is enhanced (Table 2). When DL-canaline and OAT were incubated in equimolar amounts (0.5 nmol) at 37 °C, most of the L-canaline disappeared during the first 10 s after the addition of OAT. D-Canaline reacted with the residual OAT at a lower rate.

Table 2. Inhibition of OAT by L- and DL-canaline: effects of L-ornithine and 2-oxoglutarate

Concentrations during preincubation were as follows: canaline, 10 μM; L-ornithine, 7.3 mM; 2-oxoglutarate, 1.5 mM. Residual OAT activity was determined in diluted samples as described for the time-dependent inhibition (legend to Fig. 1). The values are expressed as mean values for two separate experiments.

Time of preincubation ...	OAT activity (% of control) 0 min	30 min
DL-Canaline	68 ± 6	9 ± 2
DL-Canaline + L-ornithine	96 ± 4	87 ± 5
DL-Canaline + L-ornithine + 2-oxoglutarate	93 ± 0	47 ± 0
L-Canaline	67 ± 4	7 ± 1
L-Canaline + L-ornithine	96 ± 7	74 ± 6
L-Canaline + L-ornithine + 2-oxoglutarate	83 ± 0	29 ± 0
L-Ornithine	100 ± 4	113 ± 3
L-Ornithine + 2-oxoglutarate	100 ± 1	113 ± 5

The reaction was complete within about 20 min. OAT inhibition was reciprocal to the disappearance of canaline from the reaction mixture (Fig. 3). If DL-canaline was present in 4-fold molar excess over OAT, nearly exclusively L-canaline disappeared in a 1:1 molar ratio.

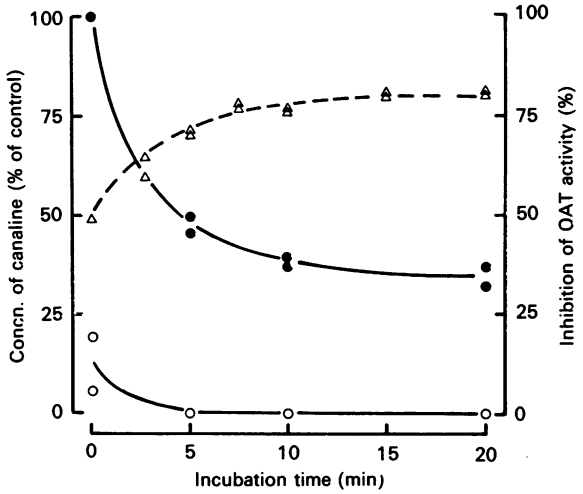


Fig. 3. Reaction of D- and L-canaline with OAT

OAT (0.5 nmol) in 50 mM-potassium phosphate buffer, pH 8.0, was incubated with 0.5 nmol of DL-canaline at 37 °C in a total volume of 250 μl. At the indicated time intervals 20 μl samples were each mixed with 20 μl of ice-cold 0.4 M-$HClO_4$. The enantiomers of canaline were determined after reaction with (+)-1-(fluoren-9-yl)ethyl chloroformate and separation of the diastereomers by h.p.l.c.: ●, D-canaline; ○, L-canaline. Residual OAT activity was determined in 200 μl samples in the absence of pyridoxal 5'-phosphate, otherwise following the description of the procedure in the Materials and methods section; the results are expressed as percentage inhibition (△).

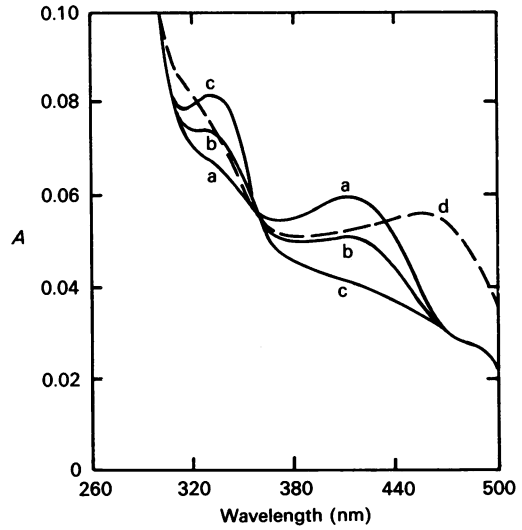


Fig. 4. Changes in the absorption spectrum of purified rat liver OAT during its inactivation by 5FMOrn

OAT (2 nmol) was mixed at room temperature with 5FMOrn (30 nmol; final concentration 50 μM) in a total volume of 600 μl of 50 mM-potassium phosphate buffer, pH 8.0. Spectra were taken at the times indicated below. Spectrum a, native enzyme (as appears from the shoulder at 330 nm, the preparation includes pyridoxamine-containing enzyme). Spectra b–d were obtained at the following times after addition of 5FMOrn: 15 s (spectrum b), 6 min (spectrum c) and 3 h (spectrum d).

Spectral changes due to inactivation of OAT by 5FMOrn

Sanada *et al.* (1976) reported a spectral shift from the maximum at 420 nm (pyridoxal 5'-phosphate band) to the pyridoxamine band at 330 nm if OAT was incubated with ornithine. The same rapid shift was observed after incubation of 2 nmol of OAT with 30 nmol of 5FMOrn (Fig. 4). A second slow shift from 330 nm to 458 nm was observed within 3 h after the addition of the

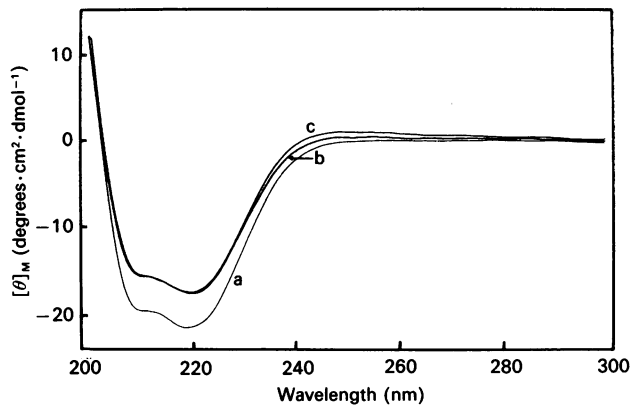


Fig. 5. C.d. spectra of a solution containing 49 μg of OAT/ml of 50 mM-phosphate buffer, pH 8.0

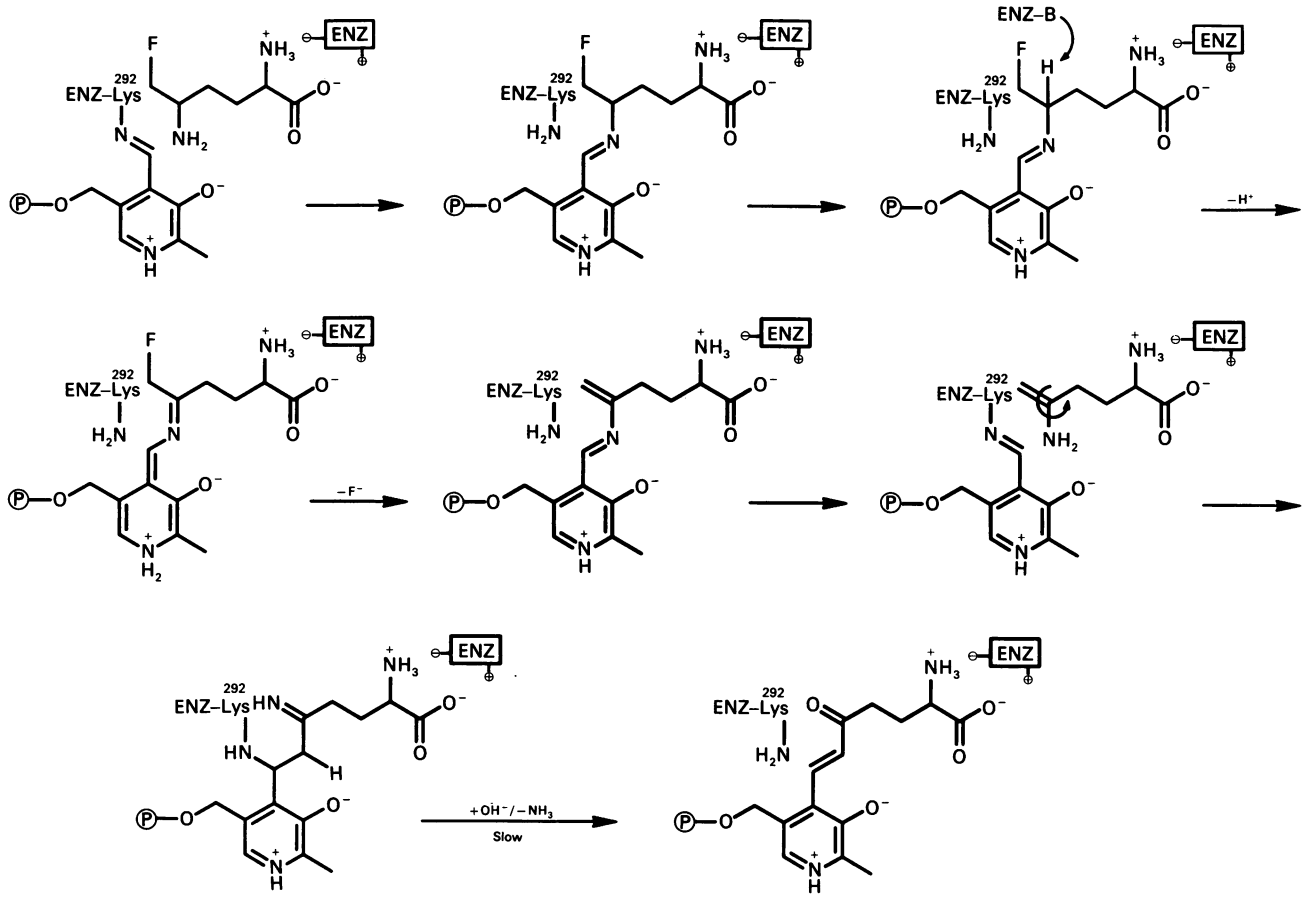
Spectrum a, enzyme after preincubation in phosphate buffer; spectrum b, enzyme after preincubation with 8 mM-5FMOrn; spectrum c, enzyme after preincubation with 8 mM-ornithine. Details of the procedure are mentioned in the Materials and methods section.

Table 3. OAT activity and ornithine concentration in liver, brain and kidney of mice after a single intraperitoneal dose of 500 mg of DL-canaline/kg body wt.

Mice were decapitated under diethyl ether anesthesia at the times indicated. The organs were isolated within less than 1 min, rinsed in ice-cold 0.9% NaCl, frozen in liquid N_2 and subsequently stored at −80 °C until analyses were performed. OAT activities and ornithine concentrations were determined from organs of the same animals. For details see the Materials and methods section. The values are expressed as means ± S.D. (n = 3–6). Abbreviation: N.D., not done.

Time after DL-canaline administration (h)	Liver	Brain	Kidney
(*a*) OAT activity (% of control)			
0	100±15	100±5	100±8
1	64±15	42±25*	8±0.5*
2	35±9*	32±3*	N.D.
4	46±5*	37±5*	15±3*
6	44±9*	38±2*	N.D.
8	39±16*	47±9*	N.D.
16	68±4*	74±8*	52±3*
24	89±20	86±4	65±2*
(*b*) Ornithine concentration (nmol/g)			
0	390±126	11±1	52±18
1	1208±294*	47±25*	167±59*
2	867±332*	78±2*	146±35*
4	526±117	44±10*	104±34*
6	598±164	37±12*	109±26*
8	704±95	29±10*	116±20*
16	511±177	20±1*	110±12*
24	410±135	12±2	54±22

* Statistically significant difference ($P \leq 0.01$) between treated and control animals (Student's t test).



Scheme 1. Postulated reaction sequence for the inactivation of OAT by 5FMOrn

inactivator. The same spectrum was obtained after extensive dialysis of the 5FMOrn-inactivated enzyme.

The c.d. spectrum of OAT exhibited only minor changes between 200 and 300 nm after reaction either with ornithine or 5FMOrn (Fig. 5).

Inhibition of OAT and accumulation of ornithine in tissues of mice after administration of DL-canaline

At 24 h after single intraperitoneal doses (10 mg/kg and 500 mg/kg) of DL-canaline no significant increases in ornithine concentrations in liver and brain of mice were observed. The time relationship of OAT inhibition after administration of 500 mg of DL-canaline/kg revealed that, even 2 h after administration of this huge dose, the inhibitor did not diminish OAT activity (as measured *ex vivo*) in brain and liver by more than 65–70% (Table 3). Subsequently the enzyme activity gradually recovered. At 24 h more than 85% of the control activity was observed. In kidney OAT activity was somewhat lower than in liver and brain, with only 8% of control activity at 1 h after the administration of DL-canaline.

The ornithine concentration followed roughly the pattern of OAT inhibition, with highest concentrations at about 2 h, and a gradual decrease to reach control values at 24 h (Table 3).

The high dose of DL-canaline necessary to elevate ornithine concentrations substantially was not without effects on the behaviour: the mice showed signs of sedation even at 24 h.

DISCUSSION

Previously published inactivation kinetic data of 5FMOrn obtained with a partially purified enzyme (Daune *et al.*, 1988) were not significantly different from the present results, which were obtained with pure OAT from rat liver.

5FMOrn is a mixture of two diastereomers, each consisting of a pair of enantiomers. By using *N*-acetyl-L-cysteine as a chiral precolumn derivative-forming reagent, it was demonstrated that only one of the four stereomers reacts with OAT. Thus it is evident that inactivation of OAT by 5FMOrn is highly stereospecific. The configuration of the active stereomer has not been established. It is, however, known that during transamination the pro-*S* hydrogen atom is abstracted from the δ-carbon of ornithine (Williams *et al.*, 1982). Moreover, only (2*S*)-ornithine is a substrate of OAT, not the (2*R*)-enantiomer. Thus we assume that (2*S*, 5*S*)-2,5-diamino-6-fluorohexanoic acid is the active enantiomer of 5FMOrn.

For (*R*/*S*)-4-aminohex-5-ynoic acid, an inactivator of both OAT and 4-aminobutyrate aminotransferase (EC 2.6.1.19) (Jung & Metcalf, 1975; Jung & Seiler, 1978), covalent binding to an amino acid residue within the active site of OAT was demonstrated (Jones *et al.*, 1983). That 5FMOrn, like ornithine, reacts first with pyridoxal 5′-phosphate is clear from the initial spectral changes and also from the fact that OAT is protected from inactivation by L-ornithine (Daune *et al.*, 1988). The absorption spectrum of OAT that is generated gradually after inactivation with 5FMOrn (Fig. 4) is different from that obtained after inactivation with (*R*/*S*)-4-aminohex-5-ynoic acid (Jones *et al.*, 1983). In the latter case the electron delocalization of the coenzyme is restricted to the pyridine ring, which results in an absorption maximum at 330 nm. After inactivation by 5FMOrn, however, the initially observed maximum at 330 nm gradually disappears and a new maximum at 458 nm becomes observable.

Extensive dialysis at pH 8.0 did not remove this slowly formed chromophore. The formation of this chromophore is in agreement with an inactivation mechanism as depicted in Scheme 1: (*a*) Schiff-base formation of the 5-amino group of 5FMOrn with pyridoxal 5′-phosphate; (*b*) β-elimination of F^-; (*c*) release of the enamine intermediate by transimination of the Schiff base to Lys-292; normally pyridoxal 5′-phosphate forms a Schiff base with Lys-292 of OAT (Simmaco *et al.*, 1986); (*d*) rotation by 180° about the C–C bond of the enamine intermediate within the active site of the enzyme; (*e*) addition of this intermediate to the Schiff base of pyridoxal 5′-phosphate; (*f*) hydrolytic release of the amino group of Lys-292; this gives rise to the formation of the chromophore with an absorption maximum at 458 nm, an unsaturated ketone.

Spectral changes similar to those observed in our work have been reported by Ueno *et al.* (1982) during inactivation of pig heart aspartate aminotransferase with serine *O*-sulphate, and a chromophore resembling the one postulated above was isolated. 4-Aminobutyrate aminotransferase is inactivated by 4-amino-5-fluoropentanoic acid, obviously following an analogous mechanism (Silverman & Invergo, 1986). The initial spectral changes observed in this case resemble our observations with OAT and 5FMOrn. The slow shift from 330 nm to 458 nm was not reported (Silverman & Levy, 1981). However, after mild alkaline hydrolysis of the inactivated 4-aminobutyrate aminotransferase a derivative of 4-amino-5-fluoropentanoic acid with a chromophore analogous to that postulated in the present paper was isolated (Silverman & Invergo, 1986).

From the absence of important changes in the c.d. spectra of OAT after reaction with 5FMOrn, changes of the protein backbone or other significant conformational changes due to inactivation can be excluded.

L-Canaline was reported to be a potent irreversible inhibitor of OAT *in vitro* as a result of oxime formation with pyridoxal 5′-phosphate (Kito *et al.*, 1978). It was found in the present work that under saturating conditions DL-canaline was as potent as L-canaline (Table 2). However, as is shown in Fig. 3, by the use of (+)-1-(fluoren-9-yl)ethyl chloroformate for separation of the enantiomers, L-canaline reacted with OAT much more rapidly than did D-canaline. At low concentrations of DL-canaline it was possible to determine the kinetic parameters of the reaction of OAT with D-canaline (Fig. 1). The reaction with L-canaline was too fast to be followed by our method. Because of the low inhibitor/enzyme ratio that inactivation by DL-canaline did not obey simple first-order kinetics, as was the case for 5FMOrn (Fig. 1).

From the fact that both enantiomers of canaline are able to form oximes with the pyridoxal 5′-phosphate of the active site of the enzyme, we conclude that the stereochemistry of C-2 of ornithine is not absolutely stringent; however, the natural L-isomer is highly favoured. The experiments performed *in vivo* showed that canaline is a poor tool for the study of consequences of OAT inhibition in the vertebrate organism.

In contrast with the expectations that were derived from the findings obtained *in vitro*, DL-canaline, if administered systemically, was a surprisingly ineffective inhibitor of OAT *in vivo*. At a dose of 500 mg/kg body wt. DL-canaline inhibited OAT maximally about 2 h after its administration, but even at this high dose inhibition was incomplete. For comparison, a dose of 10 mg of 5FMOrn/kg body wt. was sufficient to inhibit OAT by 90% (Daune *et al.*, 1988). That OAT inhibition *in vivo* was incomplete after DL-canaline administration can also be seen from the rates of ornithine accumulation in brain. After a dose of 20 mg of 5FMOrn/kg body wt. ornithine accumulated in mouse brain at a rate of 72 nmol/h per g of brain (Daune *et al.*, 1988), whereas 500 mg of DL-canaline/kg body wt. led to an accumulation rate of only 33 nmol/h per g of brain. Non-specific oxime formation with aldehydes, ketones and oxo acids, and more likely oxidative metabolism in peripheral tissues, are presumably major reasons for the inefficacy of DL-canaline *in vivo*. Intracerebroventricular injection, in contrast with systemic administration, produced a long-lasting inhibition of OAT in rodent brains (Wroblewski *et al.*, 1985; Daune, 1988).

We are greatly indebted to our colleagues Dr. T. Pelton for preparing c.d. spectra and Dr. J. Wagner for advice concerning the separation of the enantiomers of canaline.

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Received 11 August 1989/4 December 1989; accepted 19 December 1989