5-Fluoromethylornithine, an irreversible and specific inhibitor of L-ornithine:2-oxo-acid aminotransferase

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5-Fluoromethylornithine (5-FMOrn) is the first specific irreversible inhibitor of L-ornithine:2-oxoacid aminotransferase (OAT) found. Single doses (> 10 mg/kg) of this compound inactivate OAT to a residual OAT-like activity. This activity (10–20 % of total activity) is resistant to further inactivation by higher or repeated doses of 5-FMOrn, or incubation with the inactivator *in vitro*. Ornithine concentrations are greatly enhanced in various tissues, and urinary ornithine is dramatically increased, but no other amino acid is affected after acute treatment with 5-FMOrn. Repeated administration decreases carnosine and homocarnosine concentrations in brain. Toxic effects were not observed. The new inactivator is considered as a tool in the establishment of functions of OAT under physiological and pathological conditions.

INTRODUCTION

L-Ornithine:2-oxo-acid aminotransferase (OAT) is a mitochondrial enzyme present in many tissues, including liver, kidney and brain (Herzfeld & Knox, 1968). It catalyses the transamination of L-ornithine (Orn) to 2-oxoglutarate, producing glutamic acid γ -semialdehyde and glutamate (Peraino *et al.*, 1969). The liver enzyme is believed to function in the intracellular production of proline and the shuttling of carbon skeletons from excess dietary amino acids to the tricarboxylic acid cycle (Shih, 1981; Boernke *et al.*, 1981), and it was suggested that OAT competes with ornithine transcarbamoylase for Orn and thus decreases urea-cycle activity (Boernke *et al.*, 1981; McGivan *et al.*, 1977). Its function in other tissues has not yet been characterized.

The best known OAT inhibitor is L-canaline (2-amino-4-amino-oxybutyric acid), a naturally occurring structural analogue of Orn. It inhibits OAT at micromolar concentrations (Katunuma *et al.*, 1965) by forming an oxime with pyridoxal phosphate (Kito *et al.*, 1978). Thus it is not surprising that it also affects a series of pyridoxal phosphate-dependent enzymes (Kekomäki *et al.*, 1969; Rahiala *et al.*, 1971). Its biochemical effects *in vivo* therefore cannot be convincingly attributed to the inhibition of OAT (Rosenthal, 1978).

5-Fluoromethylornithine (5-FMOrn; MDL 72912) (Fig. 1) is also an analogue of Orn. It proved to be the first specific inactivator of OAT, which is sufficiently potent to achieve near-complete inhibition of OAT in all tissues of experimental animals, including the brain.

MATERIALS AND METHODS

Chemicals

If not stated otherwise, chemicals and solvents were from Merck (Darmstadt, Germany) or Baker Chemicals (Deventer, The Netherlands). 1-Chloropropanol, diethylazodicarboxylate, *N*-carbethoxyphthalimide, oxalyl chloride, triphenylphosphine, phthalimide, di-*t*-butyl dicarbonate and fluoroacetamide were from Aldrich

(Beerse, Belgium). Fluoroacetonitrile was prepared from fluoroacetamide, and 1-fluoromethylputrescine dihydrochloride (MDL 71864) was as previously described (Danzin *et al.*, 1982). The following chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.): 2-oxoglutarate, dithiothreitol, *o*-aminobenzaldehyde, L-citrulline and L-ornithine. Palladium on charcoal, and 2,4-dinitrofluorobenzene, were from Fluka (Buchs, Switzerland); *n*-octanesulphonate was from Eastman-Kodak (Rochester, NY, U.S.A.); *o*-phthalaldehyde was from Roth (Karlsruhe, Germany); and DL-[1- 14 C]-ornithine (sp. radioactivity 61 Ci/mol) from Amersham International (Amersham, U.K.). γ -Acetylenic GABA (4-aminohex-5-ynoic acid, MDL 71645) is a product of Merrell Dow Pharmaceutical Co.

Preparation of 5-fluoromethylornithine

For the structural formulae of the intermediates, see Fig. 1. All intermediates gave correct ^1H and ^{19}F n.m.r. spectra.

Fig. 1. Intermediates in the chemical synthesis of 5-fluoromethylornithine

- (II) $\text{R}_1 = \text{BzO}$; $\text{R}_2 = \text{H}$; $\text{R}_3 = \text{NH}_2$
- (III) $\text{R}_1 = \text{BzO}$; $\text{R}_2 = \text{H}$; $\text{R}_3 = \text{NPh}$
- (IV) $\text{R}_1 = \text{OH}$; $\text{R}_2 = \text{H}$; $\text{R}_3 = \text{NPh}$
- (V) $\text{R}_1 = \text{R}_2 = \text{O}$; $\text{R}_3 = \text{NPh}$
- (VI) $\text{R}_1 = \text{OH}$; $\text{R}_2 = \text{CH}=\text{CH}_2$; $\text{R}_3 = \text{NPh}$
- (VII) $\text{R}_1 = \text{NPh}$; $\text{R}_2 = \text{CH}=\text{CH}_2$; $\text{R}_3 = \text{NPh}$
- (VIII) $\text{R}_1 = \text{NPh}$; $\text{R}_2 = \text{CO}_2\text{H}$; $\text{R}_3 = \text{NPh}$
- (IX) $\text{R}_1 = \text{NHBoc}$; $\text{R}_2 = \text{CO}_2\text{H}$; $\text{R}_3 = \text{NHBoc}$
- (X) $\text{R}_1 = \text{R}_3 = \text{NH}_2$; $\text{R}_2 = \text{CO}_2\text{H}$ (MDL 72912)

Abbreviations: Boc, *t*-butoxycarbonyl; NPh, phthalimido; bz, Benzyl.

Abbreviations used: 5-FMOrn, 5-fluoromethylornithine; OAT, L-ornithine:2-oxo-acid aminotransferase (EC 2.6.1.13); GABA-T, 4-aminobutyrate:2-oxoglutarate aminotransferase (EC 2.6.1.19); THF, tetrahydrofuran; ODC, ornithine decarboxylase (EC 4.1.1.17).

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3-Benzoyloxypropyl chloride (I). To a stirred solution of benzyl bromide (0.6 mol) and 1-chloropropanol (0.6 mol) in dry THF (600 ml), potassium *t*-butoxide (0.63 mol) was added and the solution maintained below 15 °C. The mixture was stirred overnight. Acidification (1 M-HCl), extraction with diethyl ether and distillation gave (I), b.p. 76–86 °C/0.03 mmHg. [Yield 101 g (91 %).]

5-Benzoyloxy-2-aminofluoropentane (II). A solution of (I) (0.5 mol) in dry diethyl ether (500 ml) was added slowly to magnesium turnings (12.2 g) and ether (250 ml) under N₂ at a rate to maintain gentle boiling. The mixture was refluxed for 2 h and kept at room temperature overnight. A solution of fluoroacetonitrile (0.44 mol) in ether (500 ml) was added at –50 °C within 0.5 h. The stirred mixture was cooled at –60 °C. A solution of NaBH₄ (0.51 mol) in methanol (1.3 l) was added at –60 °C and water was added dropwise over 1 h. The solution was allowed to warm to 0 °C, and 6 M-HCl (300 ml) was added. After evaporation, the residue was dissolved in water (2 l) and extracted with ether. The aqueous phase was adjusted to pH 10 and extracted again. (II) was obtained as a yellow oil. [Yield 35 g (33 %).]

5-Benzoyloxy-2-phthalimido-1-pentane (III). A solution of (II) (0.16 mol) and *N*-carbethoxyphthalimide (0.15 mol) in 300 ml of benzene was stirred overnight. The precipitate was collected, dissolved in dichloromethane (400 ml), and triethylamine (69 mmol) was added. After stirring overnight the solution was washed with 6 M-HCl, dried and evaporated to give (III) as a yellow oil. Additional material was obtained by treatment of the mother liquors with triethylamine followed by chromatography on silica gel with light petroleum (b.p. 35–60 °C)/ethyl acetate (5:1, v/v). [Yield 35 g (62 %).]

5-Fluoro-4-phthalimidopentanol (IV). (III) (0.1 mol) in 250 ml of ethanol was hydrogenated at atmospheric pressure (3.4 g of palladium on charcoal) for 3 days. Chromatography on silica gel (light petroleum/ethyl acetate, 1:1, v/v) gave (IV) as a viscous colourless liquid. [Yield 22 g (87 %).]

3-Fluoro-4-phthalimidopentanal (V). Oxalyl chloride (4.3 ml; 1.1 equiv.) in dichloromethane (225 ml) was mixed under N₂ with a solution of dimethyl sulphoxide (98 mmol) in dichloromethane (225 ml) at –60 °C. (IV) (45 mmol) in dichloromethane (225 ml) was added dropwise. After stirring for 45 min at –60 °C, triethylamine (290 mmol) in dichloromethane (200 ml) was slowly added, and the temperature was allowed to rise to –10 °C. The usual work-up gave (V) as a yellow oil. [Yield 11.3 g (100 %).]

3-Hydroxy-7-fluoro-6-phthalimidohept-1-ene (VI). To a solution of (V) (88.5 mmol) in dry THF was added under N₂ dropwise a solution of vinylmagnesium bromide (0.66 mol) in THF at –78 °C. A saturated solution of NH₄Cl (200 ml) was added and the THF layer was separated. Extraction with ether, followed by chromatography [on 800 g of silica gel; eluents light petroleum/ethyl acetate (4:1, v/v), followed by light petroleum/ethyl acetate (1:1, v/v)] gave (VI). [Yield 8.4 g (34.4 %).]

7-Fluoro-3,6-bis-phthalimidohept-1-ene (VII). To a solution of triphenylphosphine (30 mmol), phthalimide (29 mmol) and (VI) (30 mmol) in dry THF (150 ml) was added under N₂ dropwise a solution of diethylazodicarboxylate (32 mmol) in 100 ml of THF. The solution was kept below 20 °C and stirred for 72 h. The usual work-up, followed by chromatography on silica gel (300 g) with light petroleum/ethyl acetate (3:2, v/v) gave (VII) as a white solid. [Yield 9.5 g (78 %).]

6-Fluoro-2,5-bis-phthalimidoheptanoic acid (VIII). A solution of (VII) (23.4 mmol) in 300 ml of acetone was stirred at 0 °C with KMnO₄ (10.9 g in 300 ml of water and 95 ml of acetic acid) for 20 h. A saturated solution of NaHSO₃ was added and the precipitate was collected by filtration. Extraction with chloroform overnight in a Soxhlet apparatus gave (VIII) as a white solid. [Yield 6.5 g (65 %).]

6-Fluoro-2,5-bis-(*t*-butylcarbamoyl)hexanoic acid (IX). (VIII) (15.3 mmol) was refluxed for 35 h in a mixture of 250 ml of conc. HCl and 90 ml of acetic acid. The solution was evaporated, the residue stirred with 100 ml of water, the phthalic acid removed by filtration and the water evaporated. The dried solid residue was extracted overnight with chloroform and gave the dihydrochloride as a white solid. This was converted into the bis-Boc derivative (IX) with di-*t*-butyl dicarbonate (57 mmol) and triethylamine (108 mmol) in 50 ml of THF and 30 ml of water (with stirring for 20 h), giving a colourless oil. [Yield 3 g (64 %).]

6-Fluoro-2,5-diaminohexanoic acid (5-FMOrn) (X). A solution of (IX) (9.7 mmol) in anhydrous ether was stirred overnight under N₂ with HCl in ether. Upon evaporation of the filtered reaction mixture, a white hygroscopic solid was obtained, which was dissolved in 60 ml of water and passed through an ion-exchange column (Dowex 50 W × 4; 200–400 mesh; H⁺ form). Elution was performed with 2 M-NH₃. The eluate was evaporated and the residue dried. Stirring with anhydrous dimethyl ether gave (X) as a very hygroscopic white solid. [Yield 0.92 g (57 %).]

Elemental analysis found: C, 42.60 %; H, 7.35 %; N, 15.86 %; C₆H₁₃FN₂O₂ · 0.25H₂O requires C, 42.72 %; H, 8.07 %; N, 16.61 %.

Conditions were chosen for isolation which avoid separation of the diastereomers. The compound gave a uniform spot on t.l.c. plates (silica gel, eluted with ethanol/25 % NH₃, 7:3, v/v; *R_f* 0.60; sprayed with ninhydrin) and one fluorescent peak (*o*-phthalaldehyde/2-mercaptopropionic acid reagent) after separation of its ion pair with octane sulphonate on a reversed-phase column under conditions that are suitable for amino acid separations (see below). ¹H n.m.r. (360 MHz; ²H₂O, reference tetradeutero-3-trimethylsilylpropionic acid): δ (p.p.m.): 1.7 (4H, m), 3.36 (1H; d of m, *J* = 23 Hz), 3.88 (1H, t, *J* = 7 Hz), 4.36 (2H, CH₂F, d of m, *J* = 47 Hz). ¹⁹F n.m.r. (338 MHz; ²H₂O, reference trifluoroacetic acid): δ (p.p.m.) (decoupled) –155.97 (s), –156.02 (s), ratio 1:1; (with ¹H coupling) d of d of t, *J*₁ = 46.6 Hz, *J*₂ = 22.8 Hz.

Laboratory animals

Male Sprague-Dawley rats (weighing at least 400 g) and female CD₁ albino mice (weighing 22 ± 2 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*) and were adapted to the laboratory conditions.

Enzymes

OAT. A partially purified preparation of OAT was obtained from livers of ten male Sprague-Dawley rats essentially by the procedure of Smith *et al.* (1977); however, the liver enzyme had not been induced by high-protein diet. Specific activity of the preparation was 7 μmol of Δ^1 -pyrroline-5-carboxylic acid formed/h per mg of protein (7 units/mg of protein).

ODC. Rat prostate was used as source of ODC. Two organs were homogenized in 5 ml of buffer (50 mM-sodium phosphate, pH 7.0, 1 mM-pyridoxal phosphate, 5 mM-dithiothreitol, 0.1 mM-EDTA and 0.2 M-sucrose). The homogenate was centrifuged (10000 *g*, 1 h), and the supernatant kept at -80°C . Specific activity was 5 nmol of $^{14}\text{CO}_2$ formed/h per mg of protein.

GABA-T. A partially purified preparation was obtained from rat brain by the procedure of John & Fowler (1976). The precipitate obtained with 45–65% satd. $(\text{NH}_4)_2\text{SO}_4$ was dialysed against 10 mM-HCl containing 10 mM-sodium acetate (adjusted to pH 7.5 with solid Tris), and the resulting enzyme solution was used.

Enzyme assays

OAT. The incubation conditions used by Strecker (1965) had to be modified to allow determination of OAT activity in brain, which requires disruption of nerve endings. The incubation mixture was as follows: 100 μl of 0.876 M-Orn (final concn. 175 mM), 100 μl of 0.176 M-2-oxoglutarate (final concn. 35 mM) (both substrates were dissolved in 50 mM-potassium phosphate, pH 8.0), 300 μl of enzyme solution (0.015 mg of protein) or the same volume of tissue homogenate in 0.5 mM-potassium phosphate buffer, pH 8.0, containing 80 μM -pyridoxal phosphate, 1 mM-dithiothreitol and 1% Triton X-100.

The mixture was incubated at 37 °C usually for 30 min with agitation. Reaction was stopped by addition of 100 μl of trichloroacetic acid (40%, v/v) and 400 μl of an aqueous solution of *o*-aminobenzaldehyde (0.125%). The colour reaction was developed by further incubation for 10 min. After centrifugation (3000 *g*, 10 min) the supernatants were allowed to cool to room temperature. They are stable for at least 24 h.

Quantitative evaluation was performed either by spectrophotometry ($\epsilon_{440} = 2700 \text{ M}^{-1}$ for the reaction product of Δ^1 -pyrroline-5-carboxylic acid and *o*-aminobenzaldehyde; Strecker, 1965) or by h.p.l.c. separation of the coloured reaction products and continuous recording of the A_{440} of the column eluate.

ODC. The activity of ODC was assayed with DL-[1- ^{14}C]ornithine as substrate, by determining $^{14}\text{CO}_2$ formation. The incubation conditions were those described by Ono *et al.* (1972); however, 1 mM acetylenic GABA was added, to avoid degradation of labelled Orn by transamination (Seiler & Sarhan, 1980).

L-Ornithine carbamoyltransferase (EC 2.1.3.3). Citrulline formation was determined by the procedure of Nakamura & Jones (1970).

GABA-T. The effect of 5-FMOrn on this enzyme was assayed essentially as described by Jung *et al.* (1977), by using the established spectrophotometric procedure for the assay of succinic semialdehyde (Scott & Jakoby, 1959; Siskin *et al.*, 1961).

Assay for time-dependent inhibition

A sample (1.12 units) of the OAT preparation (10 μl) was mixed at zero time with 240 μl of 0.2 M-potassium phosphate, pH 8.0, containing the inhibitor (and Orn, when required), and incubated at 37 °C. At certain time intervals between 0 and 30 min, 40 μl samples were diluted 20-fold, and the residual enzyme activity was assayed as described above, under standardized conditions.

Determination of polyamines

Putrescine, spermidine and spermine were determined in HClO_4 tissue extracts and urine by separation of their ion pairs with *n*-octanesulphate and subsequent reaction with *o*-phthalaldehyde/2-mercaptoethanol, as described previously (Seiler & Knödgen, 1985a).

Determination of amino acids

Tissues were stored at -80°C . Frozen samples were homogenized with 10 vol. of ice-cold 0.2 M- HClO_4 and centrifuged (3000 *g* for 15 min) after 1 h. Samples of the clear supernatants were diluted with 9 vol. of water and stored at $+2^\circ\text{C}$ until analysis; this was carried out not later than 24 h after homogenization, to minimize hydrolysis of L-glutamine. For separation by h.p.l.c., 200 μl samples of the HClO_4 extracts were applied on a Beckman Ultrasphere I.P. column. Separation was achieved by ion-pair formation with SDS and gradient elution, as described in detail previously (Seiler & Knödgen, 1985b). Our standard separation procedure allowed the determination of 5-FMOrn in brain extracts, but, owing to interfering compounds, not in liver extracts.

Selective determination of ornithine

Orn was determined as described previously (Seiler *et al.*, 1987) by treating HClO_4 tissue extracts with *o*-phthalaldehyde/2-mercaptoethanol, and separation and electrochemical detection of the isoindoles that are formed by this reaction by h.p.l.c.

Determination of 5-FMOrn

Since the racemate of 5-FMOrn is hygroscopic, it was necessary to establish precise concentrations in the solutions. This was done by diluting 10–60 μl portions of the standard solution (about 0.1 M) to 200 μl , and treating with 200 μl of a 10% (w/v) solution of 2,4-dinitrofluorobenzene in acetone in the presence of an excess of NaHCO_3 overnight at room temperature. A portion of the solution was diluted 500-fold with 0.2 M-acetic acid, and 200 μl samples were applied on a reversed-phase column (Varian Micropak MCH₅N, capped; 15 cm $\times$ 0.4 mm internal diam.). Elution was performed isocratically with methanol/0.2 M-acetic acid (81:19, v/v) (flow rate 1 ml/min; retention time 5 min). For quantification the A_{360} was recorded. Orn served as standard.

Fig. 2. Time-dependent inactivation of rat liver OAT by 5-FMOrn

(a) Rate of inactivation at inhibitor concentrations between 2.5 and 80 μM . (b) The effect of ornithine (0–10 μM) on the rate of OAT inactivation by 25 μM -5-FMOrn. The incubation and assay were carried out as described in the Materials and methods section.

Urine collection and creatinine determination

Mice were individually housed in stainless-steel metabolic cages. Urine samples were collected in glass tubes each containing 1 ml of 3 M-HCl. The volume was adjusted with water to 10 ml. Creatinine was determined in 100 μl samples by Jaffe's reaction (Loken, 1954).

RESULTS

Inactivation of OAT *in vitro*

Incubation of rat liver OAT with increasing concentrations of 5-FMOrn and subsequent determination of the residual enzyme activity revealed pseudo-first-order inactivation kinetics (Fig. 2a). From the experimental half-life ($t_{1/2}$) of the enzyme as a function of inhibitor concentration, the apparent dissociation constant ($K_i = 70 \mu\text{M}$) and the half-life at infinite inhibitor concentration ($\tau_{1/2} = 1.1 \text{ min}$) were calculated by the method of Kitz & Wilson (1962).

The inactivation rate of OAT by 5-FMOrn was decreased in the presence of Orn (Fig. 2b), demonstrating that inhibitor and substrate compete for the enzyme active site. Once the enzyme was inactivated, it could not be re-activated by extensive dialysis against 50 mM-potassium phosphate buffer (pH 8.0, containing 80 μM -pyridoxal phosphate), or by incubation with 100 mM-Orn for 30 min.

Specificity of 5-FMOrn

Incubation for 1 h of L-ornithine carbamoyltransferase, ODC and GABA-T preparations with up to 10 mM-5-FMOrn and subsequent determination of the residual enzyme activity demonstrated that none of these enzymes was inactivated. Competition studies showed no effect of 5-FMOrn on the rate of Orn carbamoylation, even with a 10-fold excess of the inhibitor; however, it decreased the rate of Orn decarboxylation competitively. From Dixon plots a K_i of 0.3 mM was determined. 5-FMOrn is a poor substrate of ODC; at 10 mM-

5-FMOrn, 0.04 nmol of 1-fluoromethylputrescine was formed/h per mg of protein. Under identical reaction conditions 4 nmol of putrescine was formed/h per mg of protein from Orn. Putrescine and 1-fluoromethylputrescine were identified and quantitatively determined by h.p.l.c. (see under 'Determination of polyamines').

Effects of 5-FMOrn *in vivo*

(a) **Single injections.** After intraperitoneal administration of 10 mg of 5-FMOrn/kg, there is a rapid decrease of OAT activity in all organs, to a basal value. Fig. 3 demonstrates the time-dependence of the effect of 5-FMOrn in brain. Minimum activity was usually observed between 2 and 24 h after 5-FMOrn administration, thereafter OAT activity recovered gradually. If we assume that OAT turnover rate is not affected by the treatment, and that the re-appearance of enzyme activity is due to enzyme synthesis *de novo*, and is not due to re-activation by removal of the inhibitor, one can calculate from our data a half-life of OAT of about 3 days for all tissues studied so far.

As shown in Fig. 3 for brain, and from results for liver and eye (not shown), intraperitoneal doses of the OAT inhibitor above 10 mg/kg did not significantly enhance further inactivation of the enzyme. In several tissues an OAT activity was observed which was refractory to inactivation by 5-FMOrn.

That treatment of mice with 5-FMOrn indeed provides the maximum possible inactivation of the enzyme was demonstrated as follows: liver and brains of mice which had been treated with 5-FMOrn (100 mg/kg) were homogenized, and the homogenates incubated for 30 min in the presence of 5 mM-5-FMOrn. Subsequent incubation with Orn demonstrated that OAT activity was not decreased by this treatment beyond the value that had been achieved *in vivo*.

The decrease in OAT activity after administration of 5-FMOrn was always accompanied by a very marked increase in tissue Orn concentrations. As expected,

Fig. 3. Effects of a single 10 mg/kg intraperitoneal dose of 5-FMOrn on OAT activity and L-ornithine concentration in mouse brain

Results are mean values ($n = 4$); the vertical bars indicate ranges (OAT activity) or S.D. (Orn concn.) respectively.

maximum Orn concentrations coincided with minimum OAT activities. However, Orn concentrations started to decrease well before a significant recovery of OAT activity was recognized. Fig. 3 and the dose/response curve (Fig. 4) demonstrate that the Orn concentrations observed in the various tissues 24 h after administration of the OAT inactivator were increasing at doses that apparently did not affect the residual OAT activity.

Orn accumulation rates were determined in mouse brain and liver as a function of the intraperitoneal dose of 5-FMOrn (Fig. 5). In brain, maximum accumulation rates were observed at intraperitoneal doses of the inactivator > 20 mg/kg. According to our experimental data, Orn is metabolized in mouse brain by transamination at a rate of $\geq 1.2 \pm 0.12$ nmol/min per g.

In liver, Orn accumulation rates are higher than in brain by a factor of at least about 20. No plateau values were observed even at the highest dose of 5-FMOrn (100 mg/kg) that was administered (Fig. 5).

Apart from Orn, none of the amino acids that is detected in tissues by reaction with *o*-phthalaldehyde/2-mercaptoethanol was affected to a measurable extent in liver or brain of mice after a single injection of 5-FMOrn, irrespective of the dose.

Putrescine concentrations were not changed in liver or

Fig. 4. OAT activity and Orn concentration in the brains of mice 24 h after a single intraperitoneal dose of 5-FMOrn

Results are mean values ($n = 4$); the vertical bars indicate ranges (OAT activity) or S.D. (Orn concn.) respectively.

eye to a measurable extent after 10 mg of 5-FMOrn/kg; however, in brain a polyphasic change of putrescine concentration was observed, the first peak coinciding with the peak Orn value. A further increase was observed around 48 h after administration of the OAT inactivator. Thereafter putrescine concentrations declined gradually, but reached normal values only after several days (Fig. 6).

In eye and brain nanomole quantities of 5-FMOrn were observed per g of tissue up to 9 h after a single intraperitoneal dose of the compound. At 24 h, 5-FMOrn concentrations in these organs were decreased below the detection limit of the method.

(b) Chronic treatment. A group of four mice received daily 10 mg of 5-FMOrn/kg intraperitoneally for 14 days; controls were injected with 0.9% NaCl. At 30 h after the last dose of the OAT inactivator, the organs

Fig. 5. Rate of Orn accumulation in mouse brain and liver as a function of the intraperitoneal dose of 5-FMOrn

Results are mean values $\pm$ S.D. ($n = 4$); the vertical bars indicate S.D. Orn accumulation rates were calculated from the increase in Orn concentration during the first 5 h after administration of the OAT inactivator, i.e. the linear part of the time/response curve.

Fig. 6. Brain putrescine (Put) concentration in mouse brain after a single intraperitoneal 10 mg/kg dose of 5-FMOrn, in comparison with the Orn concentration

Results are mean values $\pm$ S.D. ($n = 4$); *statistically significant ($P \leq 0.05$) elevation of the putrescine concentration above control value.

were isolated and analysed for a number of cell constituents. Results are summarized in Table 1. Residual OAT activity was between 21 % (brain) and 27 % (eye). The observed Orn and putrescine concentrations were within the range found after administration of single doses of 5-FMOrn, and most amino acids were unaffected by the treatment; however, there were some exceptions: carnosine, and most conspicuously homocarnosine, concentrations were decreased (Table 1).

Analyses of the 24 h urine samples demonstrated again the high specificity of 5-FMOrn. Among the usual amino acids, only Orn was markedly increased (Table 2), and in addition a small increase in putrescine, but not in spermidine and its derivatives, was observed.

Animals treated chronically with 5-FMOrn did not show any apparent deviation from normal behaviour. Thus it is unlikely that the compound is markedly toxic.

DISCUSSION

The experimental results presented in this work are compatible with the suggestion that 5-FMOrn is a potent and essentially also a specific irreversible inactivator of OAT, both *in vitro* and *in vivo*.

Decarboxylation of 5-FMOrn to 1-fluoromethyl-putrescine (5-fluoropentane-1,4-diamine), which can be further metabolized to 4-fluoromethyl-4-aminobutyric acid, may affect its specificity, since these compounds are known inactivators of ODC (Danzin *et al.*, 1982) and GABA-T (Bey *et al.*, 1981) respectively. However, the poor substrate properties, the competition with Orn and the low ODC activity of most tissues suggest that side-effects due to decarboxylation of 5-FMOrn will be an exception rather than a rule. The selective increase in Orn in brain, eye, liver and urine, without any measurable effect on brain and urinary 4-aminobutyrate concentrations, demonstrates very clearly that GABA-T

Table 1. Some biochemical effects of daily administration of 5-FMOrn for 14 days in brain, liver and eye

Groups of four female CD₁ mice received daily either 0.9% NaCl (control) or a solution (1 mg/ml; 10 mg/kg) of 5-FMOrn hydrochloride (intraperitoneally). At 30 h after the 14th dose, organs were quickly isolated and frozen in liquid N₂ until analysis. Results are mean values $\pm$ S.D.; * statistically significant ($P \leq 0.01$) difference between treated and control animals. Abbreviation: b.d.l., below detection limit of the analytical method.

Organ		OAT activity (% of control)	Ornithine (nmol/g)	Putrescine (nmol/g)	Carnosine (nmol/g)	Homocarnosine (nmol/g)
Brain	Control	100 $\pm$ 7	17 $\pm$ 4	12 $\pm$ 1	100 $\pm$ 10	180 $\pm$ 42
	Treated	21 $\pm$ 3	330 $\pm$ 130*	41 $\pm$ 9*	56 $\pm$ 15*	36 $\pm$ 5*
Liver	Control	100 $\pm$ 20	1110 $\pm$ 780	10 $\pm$ 2	32	b.d.l.
	Treated	23 $\pm$ 8	2850 $\pm$ 590	13 $\pm$ 6	29 $\pm$ 12	b.d.l.
Eye	Control	100 $\pm$ 6	220 $\pm$ 16	9 $\pm$ 1	b.d.l.	b.d.l.
	Treated	27 $\pm$ 1	728 $\pm$ 116*	14 $\pm$ 4	b.d.l.	b.d.l.

Table 2. Urinary constituents after long-term administration of 5-FMOrn to mice

Two animals per group received, instead of drinking water, a solution of 6.25 mg of 5-FMOrn/100 ml; at an average daily water intake of 5.5 ml and an average body wt. of 25 g, the animals ingested about 14 mg of 5-FMOrn/kg per day. Values are means $\pm$ S.D. of the results obtained for urine collected on days 6 and 7 of treatment.

	Creatinine (mg/24 h)	Urea (mmol/mg of creatinine)	Ornithine (nmol/mg of creatinine)	Putrescine (nmol/mg of creatinine)
Control	2.70 $\pm$ 0.27	1.02 $\pm$ 0.18	115 $\pm$ 20	85 $\pm$ 4
Treated	2.42 $\pm$ 0.36	0.99 $\pm$ 0.10	255 $\pm$ 60	114 $\pm$ 15

remains unaffected. Proline could not be determined by our method.

Studies of the mechanism of inactivation have not yet been carried out, but the available information supports the view that 5-FMOrn is an enzyme-activated irreversible inhibitor (Seiler *et al.*, 1978) of OAT. One may assume that the reactions involved in the inactivation of OAT by 5-FMOrn are analogous to those suggested by Bey *et al.* (1981) for the inactivation of GABA-T by some ω -fluoromethyl derivatives of β -alanine and 4-aminobutyrate, because OAT and GABA-T are enzymes with comparable mechanism (Williams *et al.*, 1982; Metcalf, 1979). It is therefore not surprising that some inactivators of GABA-T are also potent inactivators of OAT (Jung & Seiler, 1978; Yasuda *et al.*, 1980; Jones *et al.*, 1983). 2-Fluoromethylornithine and 2-difluoromethylornithine are enzyme-activated irreversible inhibitors of ODC (Bey, 1978).

Purified OAT from rat brain was reported to be able to catalyse the reaction between lysine and 2-oxoglutarate at a rate of about 40% of that observed for Orn (Desmukh & Srivastava, 1984). Brain lysine concentrations, however, were not affected by 5-FMOrn under conditions of near-complete inhibition of OAT, indicating that lysine transamination by OAT is quantitatively unimportant *in vivo*.

After administration of 5-FMOrn, tissue concentrations of Orn increased very rapidly, and maximum values coincided with minimum activity of OAT. However, it was observed that tissue Orn decreased already at a time when the *in vitro* OAT activity measurable, which was resistant to inactivation by 5-FMOrn ('residual OAT

activity'), was still low. Furthermore, Orn concentrations increased concomitantly with the dose of 5-FMOrn even in a dose range where no significant change in OAT activity was noticed (Fig. 5). It is assumed that the residual OAT can degrade a high proportion of the endogenously formed Orn. There is now evidence that 5-FMOrn is a competitive inhibitor of the residual OAT. In agreement with this idea, Orn concentrations remained high, as long as significant concentrations of 5-FMOrn were detectable in the tissues.

The rate of Orn accumulation in mouse brain has previously been determined after administration of maximum tolerable doses of gabaculine (Daune & Seiler, 1988), a compound which is an inactivator of both OAT (Jung & Seiler, 1978) and GABA-T (Rando & Bangerter, 1976). The value found in the present work is about twice as high as that found previously (Daune & Seiler, 1988) demonstrating the rapid and extensive inactivation of OAT by 5-FMOrn. Nevertheless, the actual rate of Orn metabolism via ω -transamination in brain may be considerably higher for the following reasons. (a) In view of the existence of the 5-FMOrn-resistant OAT activity, one has to assume a residual Orn degradation via transamination. (b) Some Orn is shifted into other pathways, such as decarboxylation to putrescine. This portion is, however, presumably small. (c) The proportion of Orn escaping from the brain via the circulation and the cerebrospinal fluid is unknown, but it may be significant.

Thus not only is this method analogous to the method of 4-aminobutyrate turnover measurements, by using GABA-T inhibitors (Van Gelder, 1966; Bernasconi

et al., 1982), but also it suffers from the same shortcomings. Nevertheless, our data at least provide the best possible approximation to Orn metabolic rates in brain *in vivo*.

Orn accumulation rates in liver were much higher than in brain. They are compatible with the higher OAT activity (Peraino & Pitot, 1963) and the higher Orn concentration (172 ± 19 nmol/g) in this organ. It is especially important in this case to remember that the experimental data are minimum rates, since, in comparison with brain, the exchange of compounds between liver and circulation is more free, owing to the absence of a specific blood/organ barrier.

That concentrations of putrescine in brain, but not in liver and eye, increased after 5-FMOrn administration is probably also due to the existence of the blood/brain barrier, which is high for the polyamines (Shin *et al.*, 1985). Moreover, in liver, ODC is assumed to be saturated with its substrate, so that further elevation of the Orn concentration is not expected to have an effect on the rate of putrescine formation. However, one has to assume that ODC in mouse brain, with a K_m of 215 ± 16 μ M (Laitinen *et al.*, 1982), is not saturated by Orn at physiological concentrations (17 μ M), hence the enzyme reaction rate will increase according to Michaelis-Menten kinetics with increasing substrate concentration. Treatment of mice with vigabatrin (4-aminohex-5-enoic acid), a specific inactivator of GABA-T (Jung *et al.*, 1977), has previously been shown to increase brain putrescine beyond the time of elevated ODC activity (Seiler *et al.*, 1979). Since we know now that elevated brain 4-aminobutyrate concentrations are accompanied by elevated Orn concentrations (Seiler *et al.*, 1987), it is likely that in both cases the same mechanism underlies the elevation of brain putrescine concentrations.

The very extensive decrease in carnosine and homocarnosine concentrations in brain after prolonged inhibition of OAT (Table 1) cannot yet be explained. Specific studies on the role of Orn as precursor of proline in brain and as substrate of carnosine synthetase are needed.

It appears that 5-FMOrn is the first specific irreversible inactivator of OAT to be found; all observations made so far with this compound support this view. 5-FMOrn may therefore prove to be a useful tool in the study of the role of Orn transamination in a variety of physiological and pathological conditions. The present results support the view that, from a quantitative point of view, Orn ω -transamination is an important pathway in different organs, including the brain. However, it is not a vital pathway, since long-term inhibition of OAT seemed not to have any apparent detrimental effects on the physiology or the behaviour of mice.

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