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7-Ethoxycoumarin Deethylation in Rainbow Trout (*Salmo gairdneri*)

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In fish, the participation of mixed function oxidases in xenobiotic biotransformation is now well known (CHAMBERS & YARBROUGH 1976). 7-ethoxycoumarin deethylation is one of the activities usually chosen to study the properties of these systems (ULLRICH & WEBER 1972 ; AITIO 1978). The purpose of this paper is to use a simple method for testing 7-ethoxycoumarin deethylation and to describe some characteristics of this enzyme in rainbow trout (*Salmo gairdneri*).

MATERIALS AND METHODS

Rainbow trouts (1-2 years old) were bred under standard conditions and killed by a blow on the head. Liver and other organs were immediately removed, weighed and rinsed with cold KCl 1.15 %. Organs were homogenized with a Potter homogenizer (or a Virtis ® cutting homogenizer) to make a 20 % homogenate. After centrifugation (10,000 g x 10 min) and filtration on glass wool, the post mitochondrial supernatant was obtained and centrifuged (100,000 g x 40 min at 4°C). The pellet was resuspended in the same volume of KCl 1.15 %, and this microsomal fraction was centrifuged again (100,000 g x 40 min , at 4°C). The pellet was resuspended in 0.25 M sucrose, 10 mM phosphate buffer pH 7.5, to obtain washed a microsomal fraction (0.8 g organ per mL) which was stored at -30°C, or at 4°C if used the same day). Protein content was determined according to HARTREE (1972).

The method used to measure 7-ethoxycoumarin deethylation was adapted from AITIO (1978). The following medium was used : phosphate buffer 0.05 M pH 7.7 ; 7-ethoxycoumarin 0.1 mM ; NADP 0.25 mM ; glucose-6-phosphate 2.5 mM ; $MgCl_2$ 1.5 mM (final concentrations) ; glucose-6-phosphate dehydrogenase 3 units ; in a total volume of 2.05 mL. The reaction was initiated by 0.2 mL microsomes (or 0.5 mL homogenate). Incubation (8 min at 30°C) was stopped by 0.5 mL TCA 1.5 M. After centrifugation (10,000 g x 8 min), 1 mL was taken and added to 2 mL NaOH-glycine buffer 2.6 M pH 10.4.

The amount of 7-hydroxycoumarin (7-OH-C) was measured with a JOBIN-YVON JY3D spectrofluorimeter (λ exc : 371 nm ; λ em : 457 nm) NADP, G6P, G6P-DH were purchased from BOEHRINGER FRANCE. 7-ethoxycoumarin (7-EC) was synthetized by RIVIERE (working in the same research station as the author) from 7-OH-C (MERCK, Germany).

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Spectral determinations were carried out with a PYE UNICAM SP8-200 spectrophotometer. Cytochrome P450 concentration was determined according to ESTABROOK & WERRINGLOER (1978), using an extinction coefficient of 91 mM^{-1} cm^{-1}. Difference spectrum was performed with the following mixture : 1.5 mL hepatic microsomes, 1 mL phosphate buffer 0.6 M pH 7.5, 4 mL water. After distribution in each cuvette (3 mL), 1 to 20 µL water (reference cuvette), or 7-ethoxycoumarin 19.02 mg/mL in water (sample cuvette) were added. A correction was made with the 7-ethoxycoumarin absorption spectrum under the same conditions.

RESULTS

The linearity of reaction was good with time up to 10 min., and with microsomes concentration up to 4.5 mg protein per incubation tube. The chosen temperature was 30°C: an assay gave a specific activity of 45 pmoles/g protein x min at 25 and 30°C, against 35 and 25 pmoles/g protein x min at 20 and 15°C respectively. The influence of pH is shown in figure 1.

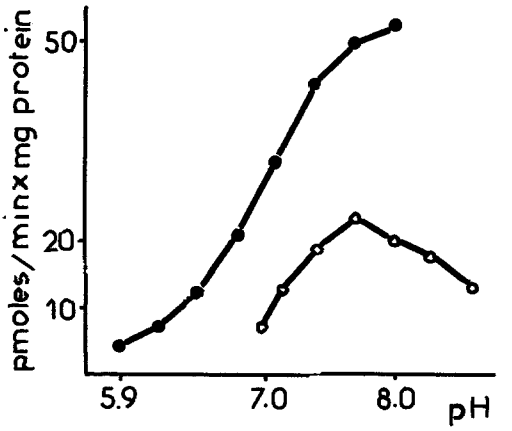

Fig. 1 - Effect of pH on 7-EC deethylation by liver microsomes
● : phosphate buffer 0,05M
o : tris buffer 0,1M

With tris buffer the optimal pH was about 7.7, but with phosphate buffer the activity was higher and had no optimal value, thus pH 7.7 in phosphate buffer was chosen for standard method.

The degradation of 7-hydroxycoumarin during incubation : evaluated in a test appeared to be quite negligible under our conditions.

TABLE 1 - Effect of cofactors on 7-EC deethylation

Cofactors in the medium	pmoles 7-OH-C / mg protein x min
NADP : 0.5 mM, G6P : 5 mM, $MgCl_2$: 3 mM G6P-DH : 5 U	30
NADP : 0.25 mM, G6P : 2.5 mM, $MgCl_2$: 1.5 mM G6P-DH : 3 U	40
NADP : 0.1 mM, G6P : 1 mM, $MgCl_2$: 0.6 mM G6P-DH : 1 U	27
NADP : 0, G6P : 0, $MgCl_2$: 0 NADH : 0.25 mM , G6P-DH : 0	0.7
NADP : 0.25 mM, G6P : 2.5 mM, $MgCl_2$: 1.5 mM NADH : 0.25 mM, G6P-DH : 3 U	47

As shown in table 1, NADPH or NADPH generating system was necessary for 7-ethoxycoumarin deethylation. NADH alone allowed for a negligible activity, while with the NADPH generating system we observed a slight synergistic effect. Optimal concentration of NADPH generating system was that with NADP 0.25 mM.

The determination of Km (fig. 2) showed a biphasic reaction and gave two values for Km (15.8 ± 0.7 µM and 75 ± 10 µM) and for Vmax (130 and 230 pmoles/mg protein x min) according to the method of calculation of WILKINSON (1961), which suggested the existence of two forms of enzyme in trout liver for 7-ethoxycoumarin deethylation.

7-ethoxycoumarin combines with hepatic microsomes to produce a difference spectrum with a peak at λ = 418 nm. This spectrum is related to a reverse-type I spectrum, but with disturbance by 7-ethoxycoumarin absorption. By plotting the reciprocal of Δ O.D. (spectral change between λ = 418 nm and λ = 500 nm) against the reciprocal of the substrate concentration (fig. 3), we obtained for Ks a value about 0.3 mM.

The distribution of 7-ethoxycoumarin deethylation in the different subcellular fractions is shown in table 2. Most of the activity occured in the microsomal fractions.

The difference in activity between the post mitochondrial supernatant (P.M.S) and the first microsomal fraction can be explained either by a lesser stability in P.M.S., or by an artifact in the analysis, or by a slight inhibition. The cytochrome P450 content was 9.5 nmoles/g liver in P.M.S., and 7 nmoles/g liver in the second microsomal fraction (73.7 % of P.M.S. content).

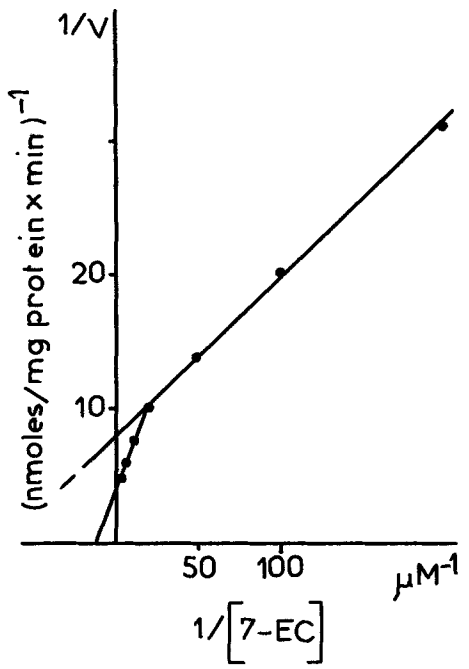

Fig. 2 - Km determination for 7-EC deethylation

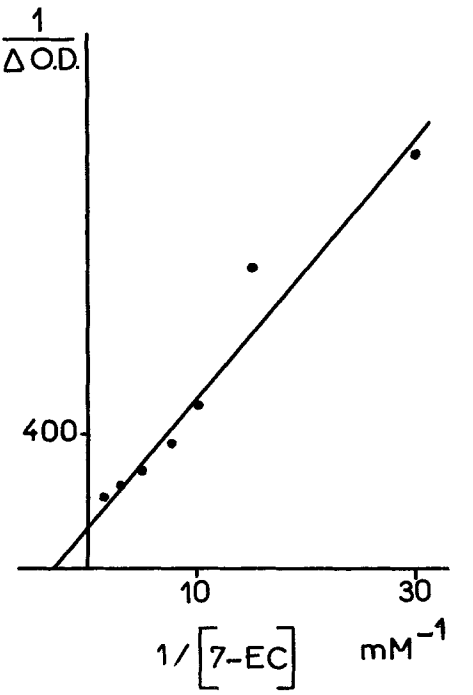

Fig. 3 - Ks determination by 7-EC difference spectrum
Δ.O.D : difference of absorbance beetween λ=418nm and λ=500nm

TABLE 2 - Activity of 7-EC deethylase in the different subcellular fractions of liver (mean of two determinations)

Fraction	pmoles 7-OH-C / g liver x min	pmoles 7-OH-C / mg protein x min
Homogenate 20 % in KCl 1.15 %	3,370	42
Post mitochondrial supernatant (10,000 g x 10 min)	2,480	44
Pellet *	790	26
1st microsomal supernatant (100,000 g x 40 min)	18	0.5
Microsomes *	2,820	150
2nd microsomal supernatant (100,000 g x 40 min)	not detected	n.d.
Washed microsomes *	2,120	130

* resuspended in the same volume of KCl 1.15 %

TABLE 3 - Localization of 7-EC deethylation in different organs

Trouts	Organ	pmoles / g organ x min	pmoles / mg protein x min	pmoles / whole organ x min
1 male (1,032 g)	liver (homogenate 20 %)	1,960	18	14,650
	gills (homogenate 20 %)	30	0.5	480
	heart (homogenate 20 %)	40	0.9	100
	kidney (homogenate 20 %)	not detected	n.d.	n.d.
	oesophagus - stomach (homogenate 20 %)	n.d.	n.d.	n.d.
	posterior alimentary canal (homogenate 20 %)	n.d.	n.d.	n.d.
	spleen (homogenate 20 %)	70	1	140
3 trouts (463 g, 528 g, 572 g) organs mixed	livers (homogenate 20 %)	2,100	23	35,800
	gills (homogenate 40 %)	23	0.6	400
	hearts (homogenate 25 %)	90	2.5	250
	spleens (homogenate 25 %)	40	4	70

As shown in table 3, 7-ethoxycoumarin deethylation was mainly located in the liver. Gills, heart, and spleen showed also some activity, but at a very low level.

Specific activity and Km of 7-ethoxycoumarin deethylation in rainbow trout liver were near those found by ADDISON *et al.* (1977) with brook trout (*Salvelinus fontinalis*). Specific activity was about the same as that found in rats without pretreatment (ULLRICH *et al.* 1975), and lower than that in little skate, *Raja erinacea* (TEND *et al.* 1976), in mice (ULLRICH & WEBER 1972), in partridge liver (RIVIERE 1980). The Km was higher in trouts than in mice (ULLRICH & WEBER 1972) and in partridge liver (RIVIERE, 1980), which means that affinity between monooxygenase and 7-ethoxycoumarin is lower.

The binding with the substrate is quite different : in rats (ULLRICH *et al.* 1973) and in partridge (RIVIERE 1980) the difference spectrum is of a type I and Ks is similar to Km. AHOKAS *et al.* (1976) showed that different substances like hexobarbital, DDT, piperonyl butoxide which produce type I spectra in rat liver microsomes did not give any spectra, or gave unclassified spectra with trout liver microsomes. Further work is necessary to better characterize the properties of mixed function oxidases in rainbow trout and their role in drug metabolism.

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