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7-ETHOXYCOUMARIN DEALKYLASE AND CYTOCHROME P-450 FROM GREY PARTRIDGE (*Perdix perdix*) HEPATIC AND DUODENAL MICROSOMES

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SUMMARY : Hepatic and duodenal microsomes were prepared from partridge by conventional procedures. The duodenal homogenates were stable, avoiding the use of protease inhibitors in the preparation of microsomes. Both microsomal fractions were able to dealkylate 7-ethoxycoumarin, showing the characteristics of a cytochrome P-450 dependent reaction. Parameters of the reaction (cofactor requirements, optimal pH, Km) were established. Typical type I difference spectrum was obtained upon addition of 7-ethoxycoumarin to hepatic and duodenal microsomes ; with liver, Km and Ks values were similar. The concentration of cytochrome P-450 was very high and similar in both organs, but the specific activity of duodenal 7-ethoxycoumarin dealkylase was about 10 % and NADPH-cytochrome c reductase 50 % that of liver.

INTRODUCTION

The cytochrome P-450 system has been mostly studied with liver microsomes. Difficulties were encountered in preparing microsomal fractions from the small intestine (duodenum), and only recently rat microsomes with stable cytochrome P-450 were prepared (1), and rabbit cytochrome P-450 purified (2). In rodents, catalytic activity and cytochrome P-450 content were usually low (3,4), the highest value was obtained from rabbit (≃ 0.6 nmol/mg protein) and represented about 40 % of the concentration in the liver (5).

We have recently reported a high aldrin epoxidase activity in the duodenum of the Japanese quail (*Coturnix coturnix*) and suggested that intestinal metabolism is a major route of detoxication in birds (6,7). We report here data about microsomal fractions from partridge (*Perdix perdix*) that confirm the previous findings. Furthermore, the duodenal microsomes from partridge have the following interesting features : they are easy to prepare, stable, and contain a very high concentration in cytochrome P-450.

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MATERIALS AND METHODS

The animals were one year-old females, reared in cages. After decapitation, liver was removed, weighed and perfused with KCl 1.15 % (all operations were performed at + 4°C).

The duodenum was excised, quickly rinsed with tap water, then with KCl 1.15 % and weighed. The liver and the duodenum were respectively homogenized with a Potter and a VIRTIS ® homogenizer to make a 20 % homogenate. After centrifugation (10,000 g. x 10 min.), the post mitochondrial supernatant was centrifuged at 100,000 g. x 60 min., then resuspended in the same volume of KCl 1.15 % and recentrifuged in the same conditions. The pellet was resuspended in 0.25 M sucrose, 10 mM phosphate buffer pH 7.5. Concentrated microsomes (800 mg organ/ml were stored at + 4°C or - 30°C.

NADP, NADPH, G6P-DH and cytochrome c were purchased from Boehringer France. Metyrapone (1,2-di-(3-pyridyl)-2-methyl-1-propanone) and 7,8-benzoflavone (α-naphtoflavone) were products of EGA-Chemie.

The O-dealkylation of 7-ethoxycoumarin was determined according to (8) and (9). The rate of dealkylation was followed at 30°C with a JOBIN-YVON JY3D spectrofluorimeter (λ exc : 371 nm ; λ em : 457 nm). The following medium was used : phosphate buffer 50 mM, pH 6.8 (liver) or 7.7 (duodenum) ; 7-ethoxycoumarin, 0.1 mM (liver) or 0.5 mM (duodenum) and 50 µl microsomes in a total volume of 3.05 ml. The reaction was started by 10 µl NADPH (final concentration : 0.05 mM for liver and 0.1 mM for duodenum).

Spectral determinations were carried out with a PYE UNICAM SP 8-200 spectrophotometer equipped with a turbid sample accessory. Cytochrome P-450 was determined according to (10), using an extinction coefficient of 91 mM^{-1} cm^{-1}. Determination of Ks was made by addition of aliquots (1-25 µl) of an aqueous (1 mM) or methanolic (100 mM) solution of 7-ethoxycoumarin to the sample cuvette.

Activity of NADPH-cytochrome c reductase was monitored continuously using the increase in absorbance of cytochrome c at 550 nm and 30°C in the following medium : phosphate buffer 65 mM (pH 7.4) ; EDTA (0.1 mM);KCN (1 mM) and 44 nmol cytochrome c in a total volume of 1.2 ml. The reaction was started by 100 µl NADPH (1 mM). Proteins were determined according to HARTREE (11) using bovine serum albumin as standard.

RESULTS

In previous reports, microsomes from duodenum were prepared by adding various protease inhibitors in homogenization medium. The most striking feature of the partridge was the possibility to obtain such a microsomal fraction without adding any inhibitor : homogenates prepared by conventional procedures exhibited no decrease in drug metabolizing activity when measured several hours after homogenization.

Microsomes from liver and duodenum were able to dealkylate 7-ethoxycoumarin to a compound with fluorescent properties and Rf values on TLC identi-

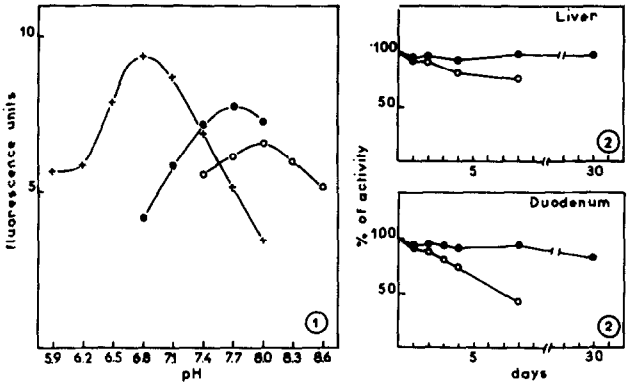
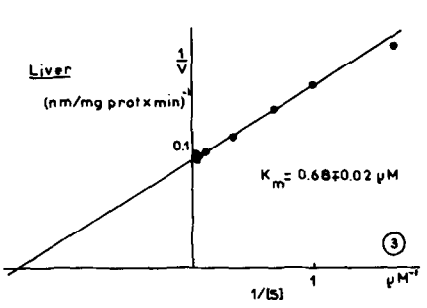


Fig. 1. Effect of pH on 7-ethoxycoumarin dealkylase (+ —— + : liver, phosphate buffer ; ● —— ●. duodenum, phosphate buffer ; o —— o : duodenum, tris buffer)

Fig. 2. Stability of microsomal fractions at + 4°C (o —— o) or - 30°C (● —— ●)

cal to 7-hydroxycoumarin. The reaction rate was linear with time, but only for a short time with duodenal microsomes. Incubation of duodenal microsomes with a NADPH-generating system showed a rapid destruction of cytochrome P-450. The reaction rate was linear with protein concentration in the range 0.2-1.5 mg. The pH optima lay around 6.8 (liver) and 7.7 (duodenum) (fig. 1). The stability of both duodenal and hepatic microsomes was good : 50 % of activity was still retained in duodenal microsomes after 1 week at + 4°C (fig. 2).

Cofactor requirements are shown in table 1 : the absolute requirement for NADPH and the inhibition by CO clearly indicated the cytochrome P-450 dependent nature of the reaction. NADH alone did not support the reaction, but caused a synergistic effect with NADPH. The reaction rate was not highly modified by EDTA, Mg^{++} or nicotinamide. The use of an NADPH-generating system instead of NADPH, gave similar results. The determination of Km gave a value of 0.7 µM (liver, fig. 3) and two values of ≃ 5 µM and ≃140 µM (duodenum, fig. 4). When 7-ethoxycoumarin was added to hepatic microsomes, a typical "type I" difference spectrum was observed with a peak at 386 nm and a trough at about 420 nm (with duodenal microsomes, a "type I" difference spectrum was also observed, but the intensity of the spectral change is considerably lower and Ks could not



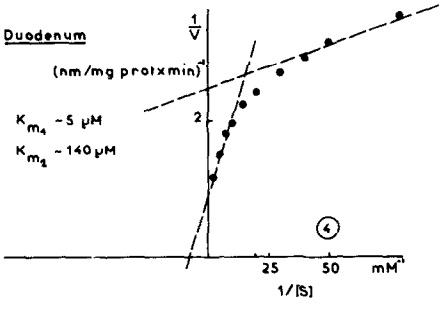


Fig. 3. Km for 7-ethoxycoumarin dealkylation by hepatic microsomes from partridge

Fig. 4. Km for 7-ethoxycoumarin dealkylation by duodenal microsomes from partridge

Table 1 : Effect of some cofactors and inhibitors on the dealkylation reaction

	liver	duodenum
Control	100	100
- NADPH	0	0
- NADPH ; + NADH (50 µM)	3	3
+ NADH (50 µM)	199	140
- NADPH ; + NADP (0.1 mM) ; G6P (1 mM); G6P-DH (1U/ml)		106
- NADPH ; + NADP (50 µM) ; G6P (0.5 mM) ; G6P-DH (1U/ml)	103	
Mg^{++} (1 mM)	103	108
EDTA (1 mM)	98	88
Nicotinamide (1 mM)	100	88
CO	14	12
Metyrapone (100 µM)	43	75
7,8-benzoflavone (10 µM)	5	53

be accurately measured).The magnitude of the spectral change was very high (Δ A 420-386 nm/Δ A P-450 : 0.37). The spectral dissociation constant (Ks) was closely related to the Km value obtained from the O-dealkylation reaction (fig. 5).

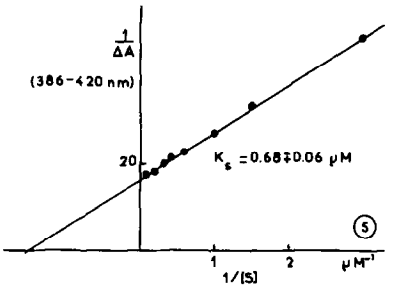


Fig. 5. 7-ethoxycoumarin difference spectrum with hepatic microsomes from partridge

Table 2 : Microsomal enzymes in hepatic and duodenal microsomes from the partridge (mean of 3 animals in each sex)

	♀		♂	
	Liver	Duodenum	Liver	Duodenum
Weight (g)	8.4	3.3	6.6	2.8
Microsomal protein (mg/g)	13.0	12.6	10.3	12.3
7-ethoxycoumarin dealkylase (nmol/mg prot x min.)	5.2	0.6	5.6	0.6
Cytochrome P-450 (nmol/mg prot)	0.45	0.49	0.40	0.44
NADPH-cytochrome c reductase (μm cyt. c red/mg protein)	0.14	0.08	0.12	0.07

The CO-difference spectrum of dithionite-reduced microsomes clearly indicated the presence of cytochrome P-450 in both duodenal and hepatic microsomes;in most instances, duodenal microsomes gave values between 449-450 nm and hepatic microsomes, values between 450-451 nm ; however, if the concentrations in cytochrome P-450 were similar, activity of the NADPH-cytochrome c reductase and 7-ethoxycoumarin dealkylase were lower in duodenum than in liver (about one-half and one-tenth, respectively) ; no significant differences were observed between males and females (table 2).

DISCUSSION

In hepatic microsomes from partridge, affinity for the 7-ethoxycoumarin and the dealkylation activity were very high, and a good correlation was obtained between Km and Ks values. Values for Km and Ks, inhibition by 7,8-benzoflavone and characteristic difference spectrum are in line with previous observations with 3-methylcholanthrene (3-MC) treated rats (12). However, the peak of the CO-difference spectrum of the reduced microsomes was found at 450 nm and the pH for maximal activity was 6.8, clearly indicating differences between microsomes from 3-MC treated rats and partridges. Moreover, unlike 3-MC treated rats, the pH/activity curve and kinetic experiments did not indicate the presence of another form of cytochrome P-450.

In duodenal microsomes, the concentration of cytochrome P-450 is very high, and apparently the partridge is one of the richest source of intestinal cytochrome P-450. Unlike similar preparations from rodents, intestinal microsomes were easy to prepare and showed negligible decrease in activity after storage. The determination of Km gave two values, thus suggesting the existence of more than one form of cytochrome P-450 in this species. Further work is now undertaken to fully characterize these intestinal microsomes and to determine the importance of intestinal biotransformation in birds.

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