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CYTOCHROME P-450-DEPENDENT O-DEALKYLASE ACTIVITY IN MAMMALIAN SKIN

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1 A modified technique for the measurement of O-dealkylase (ODA) activity in crude homogenates is reported, and its application to skin is described.

2 Large differences in ODA levels were found between different species, no activity being observed in the skin of primates.

3 Induction of cutaneous ODA in mice was achieved by the subcutaneous injection of phenobarbitone, hexachlorobenzene or 20-methylcholanthrene.

4 Attempts to induce ODA *in vitro* were unsuccessful.

Introduction

Cytochrome P-450-dependent monooxygenases catalyse the oxidation of many substances, in particular xenobiotics. This system includes several distinct enzymes, each possessing a characteristic spectrum of substrate specificities. The only member of this group which has been extensively studied in skin is aryl hydrocarbon hydroxylase (see Pohl, Philpot & Fouts, 1976, references 4–14); this latter enzyme has recently attracted attention because of the report of a systemic defect in psoriatic patients (Chapman, Kersey, Keys, Shuster & Rawlins, 1980).

Another monooxygenase which is of importance in the degradation of xenobiotics is O-dealkylase (ODA), which catalyses reactions of the type

$$R.CH_2.OR' \xrightarrow{O_2,\ NADPH} R.CHO + R'OH$$

Ullrich & Weber (1972) introduced the use of 7-ethoxycoumarin (EOC) as a convenient substrate for the measurement of ODA activity, the reaction product (7-hydroxycoumarin or umbelliferone) being highly fluorescent. Unfortunately the method advocated by these authors and used by almost all subsequent workers is unsuitable for the assay of crude tissue homogenates, being rather insensitive and requiring an optically clear solution.

More recently Greenlee & Poland (1978) have shown that the insertion of an extraction step between the incubation period and the fluorimetric measurement of umbelliferone results in a considerable improvement in sensitivity. We have therefore used this approach to investigate ODA activity in skin; our findings are presented below.

Methods

All animals used were male with the exception of 2 rabbits. Skin specimens from cattle (nose and ear) were obtained from a local slaughter-house; these were removed within 30 min of death, transported on ice and reached the laboratory within 2 h. All other animals were from stocks held by the animal-house of this University. Cattle were killed by 'pin-pistol' followed by exsanguination, rabbits by cardiac puncture and small animals by cervical dislocation. Skin was shaved where necessary and samples removed in such a way as to include all epidermal layers plus dermal adnexae (hair follicles, sweat glands etc.). In the case of mice, full-thickness abdominal skin was dissected free from fat and muscle; for other animals, split-thickness slices were removed with a keratotome (Stortz Instrument Co., USA) set for a depth of 0.5 mm.

Human biopsies were obtained from the upper back of healthy male volunteers (aged 18–24 years) by means of a keratotome after freezing the skin surface with ethyl chloride spray. For ethical reasons (avoidance of scarring) the depth was limited to 0.2 mm, which includes all epidermal layers but only part of the dermal adnexae. The monkey was biopsied as for humans but a depth of 0.5 mm was used. All specimens were assayed for ODA activity immediately or stored at −20°C for up to 4 days before analysis.

General assay procedure

This is based on the observation (Greenlee & Poland, 1978) that the reaction product may be extracted

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from aqueous acid into a non-polar solvent but returns to the aqueous phase at alkaline pH. Interfering substances remain either in the acidified reaction medium or in the non-polar solvent.

Scissor-minced tissue was homogenized in 0.1 mol/l Tris-HCl buffer (pH 7.8) at a concentration of 50 mg wet wt. tissue/ml using an ice-cooled all-glass Potter type grinder ('Microwet grinder', Townson and Mercer, Croydon); experience in this laboratory has shown this instrument to be optimal for homogenization of skin samples not exceeding 100 mg wet wt. EOC was stored as a 60 mmol/l solution in dimethylsulphoxide at −20°C, and immediately before use a portion was diluted to 6 mmol/l with Tris buffer. Aliquots of 200 μl tissue homogenate were pipetted into 4 conical glass centrifuge tubes, and 20 μl of NADPH (1 mmol/l in Tris buffer) were added to each. After bringing the tubes to 37°C, the reaction was initiated in 2 tubes by the addition of 20 μl of EOC (6 mmol/l). All tubes were incubated for 30 min, the reaction stopped by the addition of 0.5 ml 0.2 N HCl, and 20 μl of EOC subsequently added to the 2 'blank' determinations.

After cooling the tubes, 1 ml portions of ethyl acetate were added to each, the contents agitated vigorously using a 'Vortex' mixer and the phases separated by a brief centrifugation. Approximately 900 μl of the upper (ethyl acetate) layer was transferred to a clean tube, being careful not to disturb material at the interface. The remaining aqueous phase was extracted with a second 1 ml portion of ethyl acetate, and the two portions were pooled. One ml of carbonate-bicarbonate buffer (0.1 mol/l, pH 10.0) was added to the pooled extract. After mixing and centrifuging as before, a sample of the lower (aqueous) phase was transferred to a cuvette and the fluorescence measured ($\lambda_{EX} = 376$ nm; $\lambda_{EM} = 455$ nm) using a 'Fluorispec' fluorometer (Baird Atomic Europe B.V., The Hague). A solution of 10^{-7} mol/l umbelliferone in carbonate-bicarbonate buffer was used as standard. After subtraction of the blank, the umbelliferone content of the assay samples was calculated. ODA activity was expressed as fmol (10^{-15} mol) umbelliferone liberated per min and per mg fresh weight of tissue (fmol $min^{-1}mg^{-1}$).

Reaction kinetics

The kinetics of the skin enzyme were studied using an homogenate (50 mg/ml) of full-thickness skin from inbred BALB/c mice. In certain cases a liver homogenate (diluted to 2.5 mg/ml) prepared from the liver of the same animal was also examined. The effects of varying the incubation period (5–60 min), the incubation temperature (20°C and 37°C) and pH (6.5–8.7) were investigated. All measurements were performed in duplicate, and appropriate blanks were included in each experiment. The reaction conditions (other than the variable being studied) and the subsequent extraction procedures were as described above.

The stability of the skin enzyme was estimated by measurement of ODA activity of tissue stored at −20°C for periods of up to 2 weeks.

Tissue distribution and species variation

The distribution of ODA activity was investigated in various tissues of BALB/c mice. Animals were killed, shaved and dissected in the usual way. Specimens of tissue were homogenized at 50 mg/ml and ODA activity determined as described for skin, with the exception of liver homogenates, which were diluted to 2.5 mg/ml in 0.1 mol/l Tris buffer before assay.

ODA activity was determined in specimens of skin from various mammalian species which were locally available. In the case of mice, cutaneous ODA was also measured in a range of different inbred strains.

Induction experiments

These studies again employed male BALB/c mice of about 6 weeks of age. Inducing agents tested *in vivo* were phenobarbitone (PB; 80 mg/kg body weight, dissolved in phosphate-buffered saline), hexachlorobenzene (HCB; 80 mg/kg body weight, suspended in corn oil) and 20-methylcholanthrene (MC; 100 mg/kg body weight, suspended in corn oil). Both subcutaneous and intraperitoneal routes were employed. In general 2 doses were administered (0 and 24 h); animals were killed in groups of 3 at intervals up to 1 week after the first dose, and ODA activity determined in skin and liver. Appropriate controls (injecting vehicle only) were included.

Experiments were also carried out to study the effect of phenobarbitone on skin and liver maintained *in vitro*. Specimens of tissue (usually about 100 mg and 20 mg respectively) were incubated at 37°C in Eagle's minimal essential medium containing 10% foetal calf serum and 50 μg/ml gentomycin. Phenobarbitone concentrations ranged from zero to 200 μg/ml. Samples of tissue were removed at intervals up to 24 h, washed in 0.9% w/v NaCl solution (saline), and ODA activity determined in the usual way. In certain 'short' experiments the gentomycin was omitted.

Materials

EOC and NADPH were purchased from Boehringer Mannheim B.V. (Amsterdam); the EOC was used without further purification. 20-Methylcholanthrene was obtained from the Sigma Chemical Co. (USA).

Materials for tissue culture experiments were from Gibco Europe B.V. (The Netherlands). All other chemicals were of analytical quality and were purchased from Merck (Darmstadt, Germany).

Results

General

The assay procedure described here yielded a reproducibility of 12% (standard deviation of the mean for 10 duplicate samples). The overall recovery of umbelliferone standards, added before extraction, averaged 59±5% (s.d.) for a total of 15 samples over 3 separate experiments; no significant difference was found between the individual batches. The recovery was independent of umbelliferone concentration over the range 10–500 pmol/ml.

The total 'blank' fluorescence, including the contribution of the tissue itself, usually corresponded to about 1 pmol (10^{-12} mol) of umbelliferone. Thus, using an homogenate at 50 mg/ml (i.e. 10 mg tissue per tube), an incubation period of 30 min, and taking the 'detection limit' for total fluorescence as twice the blank (i.e. the corrected fluorescence of the sample equal to that of the blank), the ultimate sensitivity of the ODA assay is about 3 fmol (10^{-15} mol) min^{-1} mg^{-1} tissue.

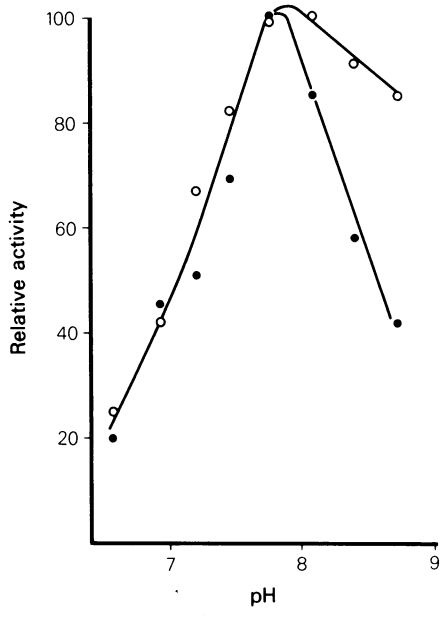

Figure 1 Effect of pH on reaction velocity of O-dealkylase in mouse liver, 2.5 mg/ml (○) and skin, 50 mg/ml (●).

Reaction kinetics of cutaneous O-dealkylase

Using an homogenate of mouse skin at 50 mg/ml, the de-ethylation of EOC was linear for 30–40 min at 37°C and for at least 1 h at 20°C. The reaction rate at the lower temperature was about one-third of that at 37°C. When a reaction time of 30 min was used the rate at 37°C was proportional to the concentration of tissue up to at least 50 mg/ml for skin and about 5 mg/ml for liver. The pH curve of cutaneous ODA (Figure 1) was similar to that of liver, showing an optimum at about pH 7.8, but fell rather more rapidly at alkaline pH values.

ODA activity was remarkably stable on storage; after 2 weeks at −20°C specimens of mouse liver and skin both retained more than 90% of their original activity.

Tissue distribution and species variation

Mouse liver was far more active than skin, averaging 13±4 pmol $min^{-1}mg^{-1}$ tissue for 6 specimens. Low and variable activity was observed in lung; no activity (i.e. < 3 fmol $min^{-1}mg^{-1}$ tissue) was found in brain, heart, intestine, kidney or spleen.

A surprisingly wide variation in cutaneous ODA levels was seen between different species of animal; activity could be detected neither in the Rhesus monkey nor in human skin (Table 1). The cutaneous ODA activity was rather similar in all strains of mice examined (Table 2).

Induction experiments

The effect of the various agents on cutaneous and hepatic ODA levels are shown in Figures 2a and b. In the cases of PB and HCB, the pooled data from days 1, 2 and 4 of each curve (i.e. 9 values) were compared with the pooled control levels by the Wilcoxon Signed Rank test; in the case of MC, pooled data from days 4 and 7 were used. Levels of significance are shown in Table 3.

Table 1 Basal O-dealkylase (ODA) activity in the skin of various mammals

Species	*No. of animals*	*Activity* (fmol min^{-1} mg^{-1} tissue)
Cattle (ear)	4	85±19
Mouse	29	48±23
Cattle (nose)	4	14± 9
Rat	4	12± 8
Rabbit (ear)	5	5± 3
Rhesus monkey	1	<3
Human	3	<3

Where not specifically indicated, specimens were from the flank or back. Mean values are given ± s.d.

Table 2 Basal O-dealkylase (ODA) activity in the skin of various laboratory strains of mice

Strain	*No. of animals*	*Activity* (fmol min^{-1} mg^{-1} tissue)
A/HeJ	4	43±15
BALB/c	14	50±23
B10D2	3	42±13
Hairless (hr/hr)	3	18± 8
Swiss (random)	3	61±18

Mean values are given ± s.d.

When given subcutaneously all three agents produced an increase in ODA activity in both skin and liver; the effects of MC occurred rather later than those of PB or HCB, maximal levels being found 4–7 days after the first dose. Intraperitoneal injection of PB was equally effective in the induction of hepatic ODA, but had no effect on cutaneous activity.

All attempts to induce ODA *in vitro* failed. When maintained in a tissue-culture system, the ODA activity of both skin and liver decreased exponentially with a 'half-life' of about 3 h. After 18–24 h incubation the activity of liver was invariably less than 1% of that of the fresh tissue. Neither inclusion of phenobarbitone (0–200 μg/ml) nor modification of the culture system (e.g. omission of gentomycin) had any appreciable effect on this loss of activity.

Discussion

Our results confirm the observation of Greenlee & Poland (1978) that the insertion of a 2-step extraction procedure into the fluorometric assay of Ullrich & Weber (1972) improves the sensitivity by two to three orders of magnitude. We find that ethyl acetate (being lighter than water) is rather more convenient than chloroform when measuring ODA activity in crude tissue homogenates because a more complete separation of the solvent from the particulate material can be obtained; however, the recovery which we obtain (about 60%) is inferior to that reported by Greenlee & Poland (1978). The apparent absence of NADPH depletion in our experiments is presumably because of the very low levels of ODA activity in our homogenates.

Only two previous measurements of cutaneous ODA activity have been found in the literature; both groups of workers (Pohl *et al.*, 1976 and Goerz, Vizethum & Tsambaos, 1979) used the continuous fluorometric assay to determine the enzyme in microsomal preparations. If the conversion factor quoted by the former group (1–3 mg microsomal protein per g fresh weight of skin) is used, we may

Table 3 Levels of significance for the induction of O-dealkylase (ODA) by various agents.

Induction procedure	*Skin*	*Liver*
PB s.c.	$P<0.01$	$P<0.05$
PB i.p.	NS	$P<0.01$
HCB s.c.	$P<0.01$	$P<0.05$
MC s.c.	$P<0.05$	$P<0.05$

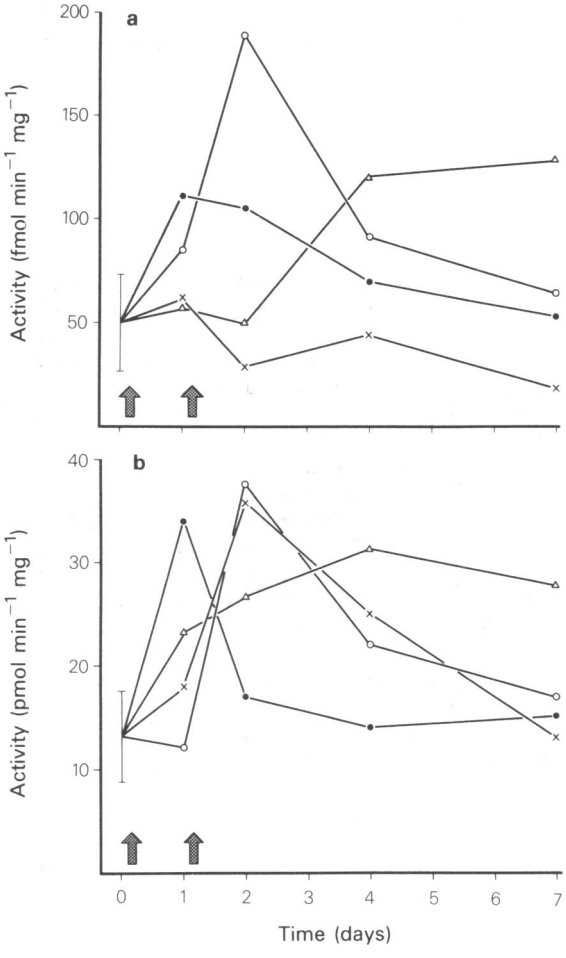

Figure 2 Effects of various induction procedures on O-dealkylase (ODA) activity in (a) skin (b) liver. The vertical bars on day zero indicate s.d. for the untreated mice, and the arrows show times of injections. Each point is the mean obtained from three animals: (○) phenobarbitone (80 mg/kg, s.c.); (●) hexachlorobenzene (80 mg/kg, s.c.); (△) 20-methylcholanthrene (100 mg/kg, s.c.); (×) phenobarbitone (80 mg/kg, i.p.).

recalculate their results to yield 80–240 fmol min^{-1} mg^{-1} for normal mouse skin (Pohl *et al.*, 1976) and about 50–140 fmol min^{-1} mg^{-1} for the skin of rats after prolonged treatment with hexachlorobenzene (Goerz *et al.*, 1979). These values are comparable with those which we obtain by direct assay of the homogenate.

Our measurements of ODA levels in the various tissues of the mouse differ in one respect from previous reports (Lehrmann, Ullrich & Rummel, 1973; Burke, Prough & Meyer, 1977), in that we were unable to detect activity in the intestine. It seems more likely that the discrepancy is related to environmental (dietary) factors than to genetic differences in the animals. The very wide variation in cutaneous ODA activity between different species (Table 1) is also surprising. Again, it is difficult to separate environmental from genetic factors; however, if these values do represent real species differences (as is suggested by the similarity of ODA levels in the various strains of mice), then we must conclude that oxidation via this pathway is not an important route in primate skin.

Previous attempts to demonstrate the induction of cutaneous ODA both used the topical route of administration. Pohl *et al.* (1976) reported induction using tetrachlorodibenzo-*p*-dioxin, but were unsuccessful using MC. Goerz *et al.* (1979) found cutaneous ODA activity after 70 days' application of HCB but could not measure pretreatment levels. In this paper we describe a convincing response, both in liver and in skin, to three different agents following subcutaneous injection. It is noteworthy that intraperitoneal administration resulted in a similar induction of hepatic ODA but had no effect on the skin, presumably because of rapid clearance of the agent from circulation by the liver. It is also of interest that the response of both tissues to MC was markedly delayed compared with PB or HCB, an observation which may indicate that it is a metabolite of MC rather than the parent compound which is responsible for ODA induction.

The remarkable decline in activity of both cutaneous and hepatic ODA *in vitro*, coupled with the failure of inducing agents to influence this process, suggest that synthesis of the P-450-mediated ODA system ceases completely under the maintenance conditions which we employ. This is in sharp contrast to the *in vitro* measurements of P-450-mediated arylhydrocarbon hydroxylase described by Chapman, Rawlins & Shuster (1979), and is surprising since changes in conditions such as oxygen tension might be expected to influence the shared electron-transport pathways rather than the substrate-specific receptors. Further work on this problem is in progress.

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