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# CYTOCHROME P-450 DEPENDENT DEETHYLASE ACTIVITY IN RAT AND HAIRLESS MOUSE SKIN MICROSOMES

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**Abstract**—Microsomal fractions were prepared from rat and hairless mouse skin. The method of preparation was validated by studying the distribution of succinate dehydrogenase, acid phosphatase and UDP-glucuronosyltransferase. Induction of oxidative deethylation activities by 5,6-benzoflavone and 3-methylcholanthrene was investigated. Preparations from hairless mouse skin exhibited higher basal activities but the enzymes were less responsive than those of rat skin to inducers. Species differences were observed in the extent of induction between topical and i.p. administration of 5,6-benzoflavone, the former route being more effective in the hairless mouse and the latter route most effective in the rat. Generally oxidative deethylation activity increased linearly with protein concn up to 2–3 mg protein/ml. The only exception was rat skin microsomes prepared from animals pretreated with 5,6-benzoflavone, where linearity was observed only to 0.75 mg protein/ml above which oxidative deethylation activity decreased with increasing protein concn. The inhibition of 7-ethoxycoumarin deethylase by various compounds was investigated; the activity in hairless mouse skin exhibited a greater sensitivity to water-soluble solvents than that in rat skin microsomes. Both hairless mouse and rat skin 7-ethoxycoumarin deethylase were sensitive to inhibition by 5,6-benzoflavone, 7,8-benzoflavone and metyrapone.

Skin is exposed, by accident or design, to a wide range of chemicals. Chronic topical application to rodent skin is routinely employed in the toxicity testing of many of these chemicals. It is known that many chemicals provoke their toxic and carcinogenic properties through conversion to active metabolites by oxidative metabolism [1] and this could occur during passage through the skin. Although many studies have been carried out on drug metabolizing activity in rodent liver, lung, intestine and kidney, very little is known about these enzyme systems in skin.

Most of the previous investigations on skin monooxygenase activity have concentrated on the metabolism of polycyclic aromatic hydrocarbons. Benzo[*a*]pyrene has been shown to be metabolized to form covalently bound adducts with cutaneous cellular constituents [2] and reports based on the measurement of the aryl hydrocarbon hydroxylase (AHH) suggests that the properties of this enzyme, both basal and induced, are similar to those of the form of cytochrome P-450 induced in liver by polycyclic aromatic hydrocarbons such as 3-methylcholanthrene [3]. However, as in liver, information based solely on measurement of aryl hydrocarbon hydroxylase activity may not be representative of total skin cytochrome P-450 mediated mixed function oxidase activity, this can only be ascertained by the use of additional substrates. The present paper details an investigation on the induction and inhibition of the skin metabolism of the model hepatic cytochrome P-450 substrates, 7-ethoxycoumarin and ethoxyresorufin. In liver, the latter has been shown to be associated with an activity that has a number of similar characteristics to benzo[*a*]pyrene 3-hydroxylase [2] while the participation of a number of forms of cytochrome P-450 in the deethylation of 7-ethoxycoumarin has been suggested [5].

## MATERIALS AND METHODS

*Chemicals.* 7-Ethoxycoumarin was prepared according to the method of Ullrich and Weber [5] and was recrystallized three times from aq methanol before use. All cofactors and 1-naphthol were obtained from Sigma Ltd. (London). Resorufin and ethoxyresorufin were obtained from Pierce Chemicals Ltd. (Chester); 7-hydroxycoumarin from Fluka A.G. (Switzerland); 5,6-benzoflavone and 7,8-benzoflavone from Aldrich Chemicals Ltd. (Gillingham, Dorset) and 1-naphthyl-β-D-glucuronide from Koch Light Ltd. (Colnbrook). The detergent Brij 35 was a gift from Dr. T. Hallinen (London). All other chemicals were of laboratory grade or analytical grade where available.

*Animals and dosage regimens.* Male Wistar rats, University of Surrey strain, and male hairless mice were used, both at an age of 45 days at which time both species were in a growth phase of their hair cycles. All animals were allowed water and a standard laboratory diet *ad libitum*.

Intraperitoneal pretreatment regimens were for 3-methylcholanthrene 20 mg/kg for 3 days, and for

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5,6-benzoflavone 50 mg/kg for 3 days, both using corn oil as a vehicle. Control animals received corn oil alone. Topical application of 5,6-benzoflavone was achieved using acetone as vehicle. Rats were clipped (Oster Clippers, Allbrooks, Rickmansworth) before treatment. An area of approx 24 sq. cm of the mid-dorsal area of the back was used for application. The dose was applied by a syringe and the animals held until the acetone was dry. 5,6-Benzoflavone (1 mg in 0.5 ml acetone) was applied by this method daily for 3 days. After topical application animals were housed separately. Animals were sacrificed by cervical dislocation 24 hr after the last topical or i.p. administration.

*Preparation of microsomes.* The dermis and epidermis were taken from the same area as that used for topical application. Any adhering fat was removed from the dermal side. Two homogenization techniques were compared, the Polytron, model PT10 (Northern Media Ltd., Hull) and Potter–Elvehjem glass homogenizer, with Teflon pestle, gap 0.01 in. driven by a Tri R motor (Rockville, U.S.A.). Before applying either of these techniques the skin pieces had to be reduced to a sufficiently small size to facilitate homogenization. Initially this was achieved by mincing with scissors. This approach was found to be unsatisfactory, especially for rat skin, where the still relatively large pieces fouled the Polytron probe and with the Potter–Elvehjem resulted in increased danger of breakage of the glass, making the procedure hazardous to the operator. Because of these factors an alternative method was adopted of freezing the skin in liquid nitrogen directly after excision and then reducing the skin to a fine powder in a pestle and mortar, keeping it frozen by the addition of liquid nitrogen to the mortar. The powder was then added to ice-cold 66 mM Tris, 1.15% KCl solution so as to yield a 15–20% homogenate. All subsequent procedures were carried out at 4°.

The typical conditions for homogenization were, for the Polytron, 10 sec at rheostat setting 2.5 and for the Potter–Elvehjem 5 passes at maximum speed Tri R drill. More vigorous homogenization conditions did not release further enzyme activity as gauged by the unaltered release of lactate dehydrogenase and glucose-6-phosphate dehydrogenase activity per g weight (data not shown). The extent of homogenization with the Potter–Elvehjem, as judged by the lactate dehydrogenase and glucose-6-phosphate dehydrognease activity, released per g wet wt of tissue, was 60% of that produced by the Polytron.

After homogenization the homogenate was centrifuged at 8000 *g* for 20 min, the supernatant decanted and centrifuged at 105,000 *g* for 1 hr to sediment the microsomal fraction. The pellet was resuspended in 0.1 M Tris–HCl buffer, pH 7.6, to the required protein concn.

*Enzyme assays.* All fluorescence measurements were carried out using a Perkin–Elmer MFP 4 fluorimeter.

7-Ethoxycoumarin was assayed fluorimetrically. The incubation mixture in a final vol. of 1 ml contained 0.1 mM 7-ethoxycoumarin, a glucose-6-phosphate dehydrogenase NADPH regenerating system (25 mM) microsomal protein ranging between 0.1 and 3.0 mg and 0.1 M Tris–HCl pH 7.6. After incubation for 20 min at 37° in a shaking water bath the reaction was terminated by addition of 0.5 ml 0.5 M glycine–TCA pH 2.2, and extracted with 4 ml ether. A 2.5 ml aliquot of this ether layer was back-extracted into 5 ml 0.2 M glycine–NaOH buffer pH 10.5 and the fluorescence in the aq phase measured at $\lambda_{ex}$ 370 nm and $\lambda_{em}$ 450 nm. Standards of 7-hydroxycoumarin (0.5–2 nmole) were taken through the entire procedure. Rates of ethoxyresorufin deethylation were assayed at 37° by the method of Burke and Mayer [4]. The incubation mixture contained 1 $\mu$M ethoxyresorufin, 250 $\mu$M NADPH, 0.1 M Tris–HCl and up to 3 mg microsomal protein. The fluorescence of the resorufin produced was monitored continuously at $\lambda_{ex}$ 510 nm and $\lambda_{em}$ 586 nm in a thermostated cuvette holder at 37°. Standards of resorufin were added to each incubation to calibrate the assay.

UDP-glucuronosyltransferase was assayed by a modified method of Bock [6] for the fluorimetric determination of 1-naphthyl glucuronide. The incubation mixture contained 5 mM UDPGA, 5 mM Mg $Cl_2$, tissue equivalent to 1.0 mg microsomal protein, 0.4 mM 1-naphthol, 0.1 M Tris–HCl buffer pH 7.6 and, when added, 0.1% w/v Brij 35 in a final vol. of 0.5 ml. After incubation for 4 min at 37° the reaction was terminated by addition of 1 ml ice-cold 0.5 M glycine–TCA buffer pH 2.2. Standards of 1-naphthyl-β-D-glucuronide were added to the appropriate tubes and the incubations were extracted with 6 ml chloroform. A 1 ml aliquot of the aq layer was added to 1 ml 1 M glycine–NaOH buffer pH 10.6 and the fluorescence read at $\lambda_{ex}$ 300 nm and $\lambda_{em}$ 334 nm.

Acid phosphatase [7] succinate dehydrogenase [8], lactate dehydrogenase [9] and glucose-6-phosphate dehydrogenase [10] were determined as previously described. Protein was assayed by the method of Goodwin and Choi [11].

## RESULTS

### *Microsomal preparation*

On comparison of the two homogenization techniques it was found that Polytron homogenization, led both to gross contamination of the microsomes with succinate dehydrogenase (>40%) and to activation of UDP-glucuronosyltransferase (data not shown) in rat skin. Both these factors indicate some pertubation or damage to cellular membranes. Neither of these effects were observed when a Polytron was used to prepare hairless mouse skin microsomal preparations. However, in contrast to the Potter–Elvehjem homogenizer, use of the Polytron resulted in a decrease in the percentage of UDP-glucuronyltransferase activity recovered in the hairless mouse skin microsomal fraction with a concomitant increase in UDP-glucuronosyltransferase activity recovered in the pre-microsomal fraction. Because of these factors Potter-Elvehjem homogenization was chosen as the most appropriate homogenization technique for the remaining part of the study.

To estimate the possibility of damage to subcel-

Table 1. Distribution of enzyme activities in hairless mouse and rat skin subcellular fractions

| | Succinate dehydrogenase | | Acid phosphatase | | UDP-glucuronosyltransferase | | Protein | |
|---|---|---|---|---|---|---|---|---|
| | Hairless mouse | Rat | Hairless mouse | Rat | Hairless mouse | Rat | Hairless mouse | Rat |
| Homogenate | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Premicrosomal fraction | 65 ± 4 | 70 ± 8 | 26 ± 2 | 24 ± 2 | 33 ± 5 | 51 ± 6 | 18 ± 6 | 21 ± 4 |
| Microsomes | 13 ± 3 | 13 ± 3 | 14 ± 2 | 20 ± 3 | 61 ± 5 | 49 ± 3 | 4 ± 1 | 7 ± 2 |
| Supernatant | 12 ± 3 | ND | 35 ± 9 | 39 ± 5 | ND | ND | 65 ± 8 | 66 ± 6 |
| Recovery | 91 ± 5 | 82 ± 6 | 76 ± 10 | 83 ± 7 | 94 ± 5 | 100 ± 4 | 87 ± 10 | 94 ± 6 |

Results are expressed as the means and standard deviations of at least three experiments of the percentage of total activity recovered in the fractions relative to the homogenate.
ND = no detectable activity.

lular organelles by the liquid nitrogen treatment, a comparison of succinate dehydrogenase distribution and latency of UDP-glucuronosyltransferase (as judged by activation by the detergent Brij 35, a widely used detergent for studying the activation of this enzyme) was made between hairless mouse skin preparations using both scissor mincing and liquid nitrogen treatment. Both preparations yielded similar results indicating that as far as could be detected, no damage to the membranes was caused by the liquid nitrogen treatment.

The quality of the microsomal fraction obtained by Potter–Elvehjem homogenization followed by differential centrifugation, was validated by the use of enzyme markers (see Table 1). Succinate dehydrogenase, a marker for the inner mitochondrial membrane, showed a similar distribution in both rat and hairless mouse skin preparations. The majority of activity was found, as expected, in the premicrosomal fraction. Acid phosphatase, a lysosomal marker, was distributed throughout the fractions. UDP-glucuronosyltransferase was used as a microsomal marker, the skin microsomes of hairless mouse and rat contained 61 and 49% respectively of this activity. Addition of the detergent Brij 35 increased the activity approx two-fold in both species. The subcellular distributions of this enzyme activity was identical both in the presence and absence of detergent (data not shown).

*Induction studies*

Ethoxycoumarin deethylase activity was found to be present and inducible in both rat and hairless mouse skin micosomes. However, preliminary experiments indicated that selection of the protein concn to be used in the incubations was important as under some conditions there was an anomalous relationship between activity and protein concn.

Figure 1 shows the relationship between 7-ethoxycoumarin deethylase activity and protein concn in rat skin microsomes. After i.p. administration of 5,6-benzoflavone to rats induction was observed and there was a linear relationship between protein

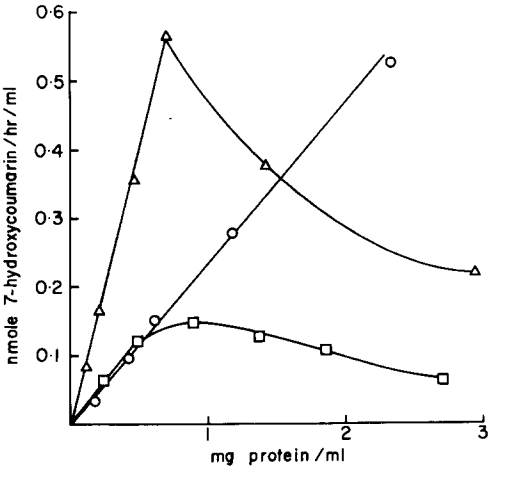


Fig. 1. Relationship between protein concn and 7-ethoxycoumarin deethylase activity in rat skin microsomes. △ 5,6-Benzoflavone-induced (i.p.); □ 5,6-benzoflavone-induced (topical); ○ 3-methylcholanthrene-induced (i.p.).

Table 2. Induction of mixed function oxygenase activities in rat and hairless mouse skin microsomes

| Species | Substrate | Corn oil | Intraperitoneal administration 3-Methylcholanthrene | 5,6-Benzoflavone | Topical administration Acetone | 5,6-Benzoflavone |
|---|---|---|---|---|---|---|
| Rat | Ethoxycoumarin | ND | 0.20 ± 0.06 | 0.76 ± 0.12 | ND | 0.24 ± 0.08 |
| | Ethoxyresorufin | ND | ND | ND | ND | ND |
| Hairless mouse | Ethoxycoumarin | 1.46 ± 0.15 | 1.45 ± 0.19 | 1.84 ± 0.19 | 1.39 ± 0.08 | 4.97 ± 1.14* |
| | Ethoxyresorufin | 1.08 ± 0.35 | † | 0.78 ± 0.30 | 0.93 ± 0.15 | 2.01 ± 0.21‡ |

Activities are expressed as nmole/hr/mg protein and are the means and standard deviations of at least three experiments.
ND = non-detectable (<0.01 nmole/hr/mg protein).
* $P < 0.01$.
† Not measured.
‡ $P < 0.005$.

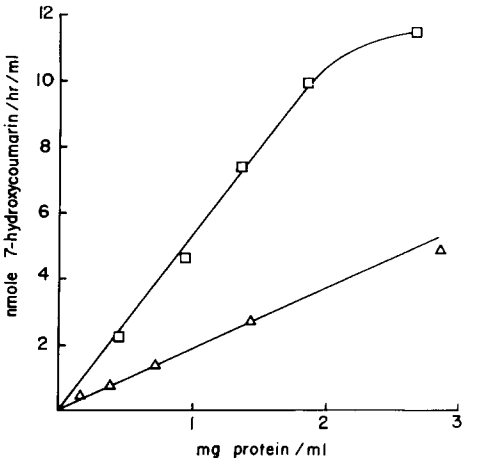


Fig. 2. Relationship between protein concn and 7-ethoxycoumarin deethylase activity in hairless mouse skin microsomes. △ 5,6-Benzoflavone-induced (i.p.); □ 5,6-benzoflavone-induced (topical).

concns and activity up to 0.75 mg protein/ml, the activity then dropped sharply with increasing protein concn. A similar effect was observed after topical administration of 5,6-benzoflavone. In contrast, after induction by i.p. administration of 3-methylcholanthrene to rats the activity was linear up to the highest protein concn tested, 2.4 mg protein/ml.

The relationship between 7-ethoxycoumarin deethylase activity and protein concn in hairless mouse skin microsomes is shown in Fig. 2. After topical induction by 5,6-benzoflavone linearity between protein concn and enzyme activity was preserved until approx 2 mg protein/ml while after i.p. induction by 5,6-benzoflavone the relationship was still linear at the highest concn tested which was nearly 3 mg protein/ml.

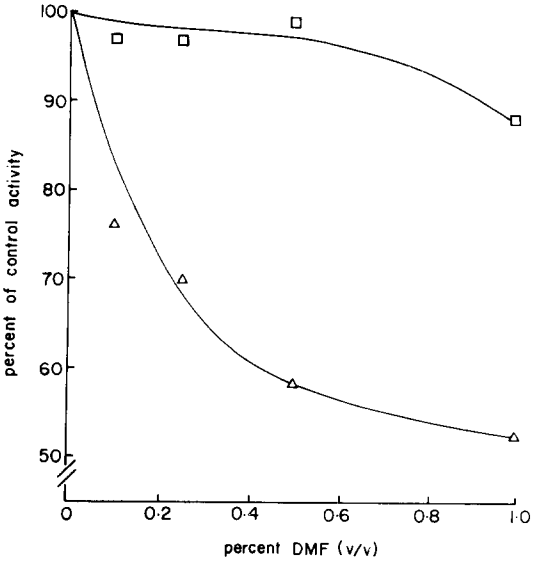


Fig. 3. Effect of DMF on 7-ethoxycoumarin deethylase activity in rat and hairless mouse skin microsomes. Points represent means of two experiments. □ Rat (3-methylcholanthrene-induced); △ hairless mouse (control).

Table 3. Effect of solvents on 7-ethoxycoumarin deethylase in skin microsomes

| Solvent | mM concn at 0.1% v/v | Percentage of control 7-ethoxycoumarin deethylase activity: Hairless mouse Control | Hairless mouse Induced | Rat induced |
|---|---|---|---|---|
| DMF | 13.0 | 75 | 82 | 96 |
| DMSO | 14.1 | 69 | 80 | 97 |
| Acetone | 13.6 | 70 | 77 | 95 |
| Methanol | 27.7 | 84 | 77 | 96 |
| Ethanol | 17.2 | 70 | 76 | 69 |

Rat induced by 3-methylcholanthrene (i.p.) and hairless mouse induced by 5,6-benzoflavone (topical).

Values are the means of two experiments.

To ascertain the cause of the anomalous effects of protein concn, in preparations from rats treated topically with 5,6-benzoflavone, on metabolism, differing amounts of 7-hydroxycoumarin were incubated under the same conditions as the 7-ethoxycoumarin deethylase assay over a range of protein concns of skin microsomes. No decrease in the amount of 7-hydroxycoumarin extracted, with increasing protein concns, was evident (data not shown), indicating that the 7-hydroxycoumarin was not being bound or further metabolized. The decreasing 7-ethoxycoumarin deethylase activity in 5,6-benzoflavone-induced rat preparations with increasing protein concn therefore appears to be due to a factor affecting the deethylation reaction rather than an artefact in measurement of the 7-hydroxycoumarin produced. As will be discussed later, it was considered that the drop in activity could be due to residual levels of 5,6-benzoflavone being present in the microsomes causing an inhibitory effect.

All subsequent investigations of 7-ethoxycoumarin deethylase in 5,6-benzoflavone-induced rat skin microsomes were carried out under conditions where the activity was related linearly to protein concns.

Ethoxyresorufin deethylase activity was not detectable in any rat skin preparations. In contrast this activity was readily detectable in both control and induced hairless mouse skin microsomes and activity in all cases was linear up to the highest protein concn tested, 3 mg protein/ml (data not shown).

Table 2 shows the control and induced deethylation activities in rat and hairless mouse skin microsomes. 7-Ethoxycoumarin deethylase activity was not detectable in skin microsomes from control rats, therefore the presence of significant activities in skin microsomes obtained from pretreated rats indicates a very considerable induction. The most effective pretreatment in rats was 5,6-benzoflavone given i.p. Topical application of 5,6-benzoflavone and i.p. administration of 3-methylcholanthrene were about equal in effect.

In contrast the hairless mouse exhibited higher basal levels but lower responsiveness to inducers than the rat. Only topical application of 5,6-benzoflavone brought about a significant rise in ethoxyresorufin deethylase, with a doubling of activity over control levels. Intraperitoneal administration of inducers had little effect on ethoxycoumarin deethylase, but topical application of 5,6-benzoflavone resulted in a 260% increase in ethoxycoumarin deethylase activity.

*Inhibition studies*

Ethoxycoumarin deethylase activity was used to investigate the inhibitory potential of a number of compounds on skin microsomal preparations. Because of the complex relationship between protein concn and activity in 5,6-benzoflavone-induced rat skin microsomes, induced skin microsomes from i.p. 3-methylcholanthrene rats were used in this study.

The effects of water-miscible organic solvents on ethoxycoumarin deethylase activity was investigated primarily because such solvents are useful vehicles for the introduction of compounds, e.g. benzoflavone with limited solubility in water, into aq incubations. Initial experiments were confined to studying the effect of dimethylformamide on control

Table 4. Inhibition of 7-ethoxycoumarin deethylase in skin microsomes

| Inhibitor | Concn (M) | Percentage of control 7-ethoxycoumarin deethylase activity: Hairless mouse Acetone (topical) | Hairless mouse 5,6-Benzoflavone (topical) | Rat 3-Methylcholanthrene (i.p.) |
|---|---|---|---|---|
| 5,6-Benzoflavone | $10^{-6}$ | 71 ± 8 | 39 ± 8 | 34 ± 16 |
| | $10^{-7}$ | 82 ± 4 | 84 ± 6 | 67 ± 10 |
| 7,8-Benzoflavone | $10^{-6}$ | 68 ± 10 | 29 ± 9 | 33 ± 10 |
| | $10^{-7}$ | 83 ± 7 | 76 ± 6 | 81 ± 18 |
| Metyrapone | $10^{-3}$ | 28 ± 7 | 18 ± 5 | 20 ± 5 |

Results are expressed as means and standard deviations of three experiments.

hairless mouse and induced rat skin microsomal activities. The rat ethoxycoumarin deethylase activity was only sensitive at the highest concn of dimethylformamide used, 1.0% v/v, whereas the hairless mouse activity was inhibited by 25% at the lowest concn used, 0.1% v/v, and plateaued at approx 50% above 0.5% v/v dimethylformamide.

A number of other solvents were then tested at a concn of 0.1% v/v on control and induced hairless mouse and rat skin.

The results are shown in Table 3. The rat skin ethoxycoumarin deethylase was insensitive to all solvents except ethanol. Enzyme activities in hairless mouse, both control and induced, were inhibited between 20 and 30% by all five solvents (dimethylformamide, dimethylsulfoxide, acetone, methanol and ethanol). These studies therefore indicate a clear difference between the ethoxycoumarin deethylase activity in hairless mouse and rat skin microsomes.

A consequence of this sensitivity to solvents of the hairless mouse ethoxycoumarin deethylase was that solvents could not be included in benzoflavone inhibition experiments with hairless mouse skin microsomes. Metyrapone studies were not compromised as metyrapone was added in an aq solution. To overcome the solvent problem with benzoflavone inhibition studies, aliquots of the compound in acetone were placed into the incubation vessels and the acetone blown off before further additions. In comparisons using rat skin microsomes administration of benzoflavone in acetone using the above procedure was compared with the direct addition of benzoflavone in acetone to microsomes in buffer. (N.B. Acetone at 0.1% v/v is not inhibitory in rat skin microsomes.) No difference in the deethylase activity was observed so introduction of the benzoflavones to the tubes and drying prior to the incubation with microsomes from hairless mouse skin was used routinely.

The results of inhibition experiments with 5,6-benzoflavone, 7,8-benzoflavone and metyrapone are shown in Table 4. 5,6-Benzoflavone and 7,8-benzoflavone showed similar inhibitor patterns. All preparations were sensitive to these isomers, induced preparations were very similar in their susceptibility to inhibition. Control hairless mouse skin microsomal activity was less sensitive to both benzoflavones at a concen of $1 \times 10^{-6}$ M, but the extent of inhibition at $1 \times 10^{-7}$ M was similar to the other preparations. Metyrapone had a similar effect on all preparations, showing strong inhibition at a concn of $1 \times 10^{-3}$ M.

## DISCUSSION

In most extrahepatic tissues subcellular fractionation procedures have not been well documented. Due to the heterogeneity of cells often found in extrahepatic tissues compared to liver and the different techniques used to homogenize these tissues, application of the differential centrifugation schemes devised for the liver must be validated for each tissue and each homogenization technique.

In common with the recent findings of Manil *et al.* [12] using mouse skin, we observed that vigorous homogenization procedures, e.g. lengthy Polytron homogenization, are not necessary for the homogenization of rat and mouse skin; indeed damage to subcellular organelles may result.

It was observed in the subcellular distribution study that acid phosphatase activity was distributed throughout the skin fractions. This has been reported previously [13]. In consequence some investigators have suggested there may be two distinct cutaneous acid phosphatase activities, one lysosomal and one cytosolic [13, 14]. However, other studies indicate that the multiple localization of acid phosphatase is most likely secondary to the disruption of lysosomes, since both lysosomal and cytoplasmic acid phosphatase have been shown to be identical by a number of parameters [15]. The percentage of acid phosphatase recovered in the skin microsomes in the present study, 14% for the hairless mouse and 20% for the rat, are similar to that reported for rat liver microsome preparations [16].

Succinate dehydrogenase, a marker for the inner mitochondrial membrane was located mainly in the premicrosomal fraction; some cross-contamination occurred for 13% of the activity appeared in the microsomes. UDP-glucuronosyltransferase was used as a marker enzyme for the endoplasmic reticulum.

Approximately half the total UDP-glucuronosyltransferase activity was located in the rat and hairless mouse skin microsomal fractions. As these activities were associated with 4 and 7% of the protein in homogenates obtained from the hairless mouse and rat respectively, they represent a considerable purification.

From the subcellular distribution studies it can be seen that the fraction designated the microsomal fraction in this investigation represented a significant purification of microsomal enzyme activity at the expense of mitochondrial and lysosomal material. However the recovery of UDP-glucuronosyltransferase in this fraction is lower than in liver where a recovery of 76% of this activity, employing 4-methylumbelliferone as aglycone, has been reported [16].

The unusual relationship between protein concn and 7-ethoxycoumarin deethylase activity in rat skin microsomes derived from 5,6-benzoflavone pretreated animals necessitated the careful choice of incubation conditions. The apparent decrease in activity observed with increasing protein concn could have been due to binding of the substrate, binding or further metabolism of the product, or inhibition of the reaction by residual amounts of 5,6-benzoflavone being present in the microsomes.

Binding or further metabolism of the product could not be detected. It is unlikely that binding of 7-ethoxycoumarin was the cause as microsomes from 3-methylcholanthrene treated rats, which might be expected to show comparable binding capacity, did not show this effect and neither did control or induced hairless mouse skin. In inhibition studies, 5,6-benzoflavone was a potent inhibitor of ethoxycoumarin deethylase; thus we consider that the non-linearity of ethoxycoumarin deethylase activity with protein concn was due to the attainment of inhibitory levels of 5,6-benzoflavone at higher protein concns. That a similar phenomenon did not occur in hairless mouse skin microsomes, despite the fact that ethoxycoumarin deethylase activity was sim-

ilar in its sensitivity to inhibition by 5,6-benzoflavone, presumably reflects a lower residual level of this compound in these microsomes. Whether the proposed residual 5,6-benzoflavone arose from cells from which the microsomes originated or was released from cutaneous lipid deposits upon homogenization is uncertain.

3-Methylcholanthrene was ineffective in inducing 7-ethoxycoumarin deethylase in hairless mouse skin microsomes in this study. This finding is in agreement with a previous report that topical application of 3-methylcholanthrene did not raise mixed function oxidase activity in hairless mouse skin [17], although 3-methylcholanthrene has been reported to induce AHH activity in a number of mouse strains that have a normal pelage [18]. Both benzoflavone isomers have also been reported to induce AHH activity in mouse skin [19], however, the relative effectiveness of topical or i.p. pretreatment of 5,6-benzoflavone on AHH induction reported for Swiss mouse [19] skin is the opposite of that observed in this study for hairless mouse skin, but similar to that observed for rat skin 7-ethoxycoumarin deethylase, that is the i.p. route brought about the greatest induction. This raises the question as to whether the differences between the induction patterns for the two routes of administration observed in this study for the rat and hairless mouse is a species difference or a difference between hairy and hairless skin, which might have particular relevance for human skin. The reason for such differences are unknown, although availability of the inducer and also distribution of activity in the skin is most likely of importance.

If there is inducible activity associated in the rat with the pilosebatory apparatus, which has a good blood supply, i.p. administration would be expected to make the inducer readily available to this site. Where the pilosebatory apparatus is lacking, as in the hairless mouse, and the monooxygenase activity [15] restricted to the epidermis (i.e. the principal site of a poor AHH activity [20, 21]) which has no direct blood supply, this may account for the poor effectiveness of an i.p.-administered inducing agent.

Control levels for rat 7-ethoxycoumarin deethylase were non-detectable and so the exact magnitude of the increase in enzyme activity in this space could not be calculated, however, it was apparent that the rat was more responsive than the mouse to the inducers employed. The greater inducibility of the activity of 7-ethoxycoumarin deethylase activity in rats than mice has also been observed in experiments on skin strips taken from these two species [22]. Despite the much greater magnitude of induction in rats the level of induced enzyme activities was still not as high as that found in control hairless mouse skin. There is therefore a marked intrinsic species difference in the microsomal P-450 activity in these two species.

In this study it was observed that both rat and hairless mouse skin 7-ethoxycoumarin deethylase were equally sensitive to 5,6-benzoflavone and 7,8-benzoflavone. In contrast, in rat liver 7,8-benzoflavone has been reported to be a more potent inhibitor of AHH than 5,6-benzoflavone [23]. The only major difference in the effects of these inhibitors between control and induced hairless mouse preparations was the greater sensitivity after induction to either benzoflavone isomer at a concn of $1 \times 10^{-6}$ M. A more comprehensive examination of the effects of the benzoflavone isomers might elucidate differences between control and induced activities. Both benzoflavone isomers have been reported to inhibit control and induced mouse skin aryl hydrocarbon hydroxylase with the control activity being slightly less sensitive [19].

Hepatic microsomal 7-ethoxycoumarin deethylase from control and phenobarbitone pretreated rats has been previously reported to be sensitive to metyrapone but insensitive to 7,8-benzoflavone [24]. 3-Methylcholanthrene induction led to a reversal of this hepatic inhibition pattern [25]. In the present study the 7-ethoxycoumarin deethylase in skin microsomes from control and 5,6-benzoflavone-induced hairless mice was sensitive to both metyrapone and 7,8-benzoflavone. The 3-methylcholanthrene induced activity in rat skin microsomes was similarly sensitive to both compounds. This indicates that there may well be significant differences between the cytochrome(s) P-450 supporting 7-ethoxycoumarin deethylation in skin and liver.

A clear difference between the 7-ethoxycoumarin deethylase activity from the skin of the two species used was shown in the response to water-miscible organic solvents, which, with the exception of ethanol, were inhibitory at very low concns to the hairless mouse activity but not to the rat activity. Rat hepatic 7-ethoxycoumarin deethylase has been reported to be inhibited by a number of water-miscible organic solvents [26], but the concns used were approximately 100-fold higher than those used in this study, making comparison difficult. It would be of interest to investigate the *in vivo* effects of these solvents as they are widely used in industry and frequently come in contact with human skin.

The findings from the present study would appear to be broadly similar to previous reports on cutaneous and hepatic AHH activity in that they are inducible by 5,6-benzoflavone and, in the case of rat 7-ethoxycoumarin deethylase, by 3-methylcholanthrene. Similarity is also shown by the sensitivity of 7-ethoxycoumarin deethylase to inhibition by 7,8-benzoflavone, but the response to metyrapone was distinctly different. This finding together with the differences in the sensitivity of 7-ethoxycoumarin deethylase to water-miscible organic solvents between rats and hairless mice would indicate that skin cytochrome P-450 dependent activities (a) are different in different species and (b) do not appear to reflect the activities in the liver of the same species.

The microsomal preparations which we have described should prove of value in investigating the relationship between drug metabolism and covalent binding in the skin.

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