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Epidermis: A Site of Drug Metabolism in Neonatal Rat Skin

Studies on Cytochrome P-450 Content and Mixed-Function Oxidase and Epoxide Hydrolase Activity

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SUMMARY

Comparative activity of drug-metabolizing enzymes, the hemeprotein cytochrome P-450 and epoxide hydrolase, in whole skin, epidermis, and dermis of neonatal rats was analyzed. For the first time, measurable and highly reproducible difference spectra were obtained from neonatal rat skin microsomes. Quantitative CO-dithionite-reduced minus dithionite-reduced difference spectra were obtained only from Aroclor 1254-treated epidermal microsomes. Cytochrome P-450 concentrations in epidermal microsomes from Aroclor-treated neonatal rats were 17.8 and 40.5 pmoles/mg of protein, respectively, when estimated using CO-dithionite-reduced minus dithionite-reduced and CO-dithionite-reduced minus CO-reduced difference spectra. After topical application of Aroclor 1254 to neonatal rats the observed percentage increases in cytochrome P-450 contents of whole skin, dermis, and epidermis were 15, 106, and 87, respectively. Aryl hydrocarbon hydroxylase, 7-ethoxycoumarin *O*-deethylase, and epoxide hydrolase activities were highest in epidermis as compared with dermis or whole skin when expressed as product per minute per milligram of protein or product per minute per gram of tissue. Induction of specific activities of epidermal aryl hydrocarbon hydroxylase (1080%), 7-ethoxycoumarin *O*-deethylase (493%), and epoxide hydrolase (21%) was observed following topical application of Aroclor 1254 to neonatal rats. When total organ activities for dermis and epidermis were added, activities approximating that of whole skin were recovered. In control animals, aryl hydrocarbon hydroxylase and 7-ethoxycoumarin *O*-deethylase activities expressed as product per nanomole of cytochrome P-450 were less than 50% of the corresponding hepatic activities, whereas in Aroclor-treated animals these activities exceeded metabolic rates in liver. These results indicate that skin has the capacity to metabolize xenobiotics and that epidermis, often considered to be metabolically inert, is an active site of such enzyme activity.

INTRODUCTION

Skin is the largest organ of the body as determined by its wet weight or by its surface area, yet the skin is frequently overlooked when assessing its participation in or susceptibility to the toxicological and pharmacological effects of environmental agents. Traditionally, skin has been regarded as a passive, inert structural barrier between the body and its environment. In recent years, as newer knowledge has developed, this concept has been modified, and it is now clear that skin also possesses enzymatic activity which is modified by exposure to endogenous and exogenous chemicals such as drugs, selected chemical carcinogens, and other environmental chemicals, including the halogenated hydrocarbons (for review see refs. 1 and 2 and references therein). Skin is a complex tissue composed of different structural components containing several types of cells. The two most important components are the epidermis and the dermis. The epidermis is the surface layer and is essentially a stratified squamous epithelium in direct and continuous contact with the environment. It consists of two major compartments, the outer stratum corneum, which is acellular and rich in the compact disulfide-rich protein keratin, and the underlying germinative layer consisting primarily of replicating keratinocytes as well as melanocytes and Langerhans' cells. The stratum corneum functions as the structural "barrier" between the body and the environment. The major evidence for this rests upon the observation that its removal is followed by greatly

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enhanced percutaneous absorption of exogenous chemicals. The second epidermal compartment consists of multiple stratified layers of cells known as keratinocytes which replicate rapidly and ultimately differentiate into the keratin-rich stratum corneum. In contrast to the intert stratum corneum, the underlying layers of keratinocytes are highly active metabolically.

Very few studies have been carried out to assess the capacity of skin to metabolize drugs and chemicals. However, it has previously been shown that the microsomal fraction of skin contains the hemeprotein cytochrome P-450 (3, 4) and that drug-metabolizing enzymes such as AHH,[1] 7-EC, epoxide hydrolase, and glutathione-*S*-transferases are present in cutaneous tissue (1–6).

A major problem that has seriously hindered the progress of research in studying drug metabolism in skin is the presence of structural proteins in cutaneous tissue that are resistant to conventional homogenizing techniques that work well to disrupt cells in other tissues. Lack of reproducible homogenization techniques has in turn complicated the development of reliable subfractionation procedures for obtaining subcellular components of this tissue. In recent years our laboratory has made a major effort to develop reproducible homogenization and subfractionation techniques that will yield microsomal fractions from skin with optimal catalytic activity for various substrates.[2] In these studies we have elected to focus on the neonatal rat as an experimental animal model, primarily because the skin of these immature animals is much less resistant to homogenization procedures. In addition, the skin of the neonatal rat is hairless, which precludes the need for potentially traumatic shaving. Since the animals are newborn, exposure to environmental pollutant chemicals which could alter activity of drug-metabolizing enzymes is minimized.

It has been hypothesized that the epidermis is the major site of drug metabolism in skin (7, 8). However, most of the studies to date have utilized a crude scalpel-scraping technique to obtain an "epidermal fraction" from rodent skin. In these studies no histological verification of the purity of the epidermal fraction has been provided. The epidermis is tightly adherent to the dermis, and separation without concomitant damage to enzymes is difficult to achieve. Other techniques for epidermal-dermal separation, including heat treatment and use of enzymes (trypsin or collagenase), have proven unsatisfactory for various reasons. Recently, however, Epstein *et al.* (9) have developed a new technique for separating epidermis and dermis using dithiothreitol. This relatively simple procedure permits complete separation of epidermis from dermis in neonatal rodent skin. Preliminary results obtained in our laboratory indicated that this new separation technique did not alter the activity of drug-metabolizing enzymes. In this paper this technique has been utilized to assess carefully the comparative activity of microsomal drug-metabolizing enzymes, the hemeprotein cytochrome P-450, and epoxide hydrolase in epidermis and dermis of neonatal rat skin.

[1] The abbreviations used are: AHH, aryl hydrocarbon hydroxylase; 7-EC, 7-ethoxycoumarin *O*-deethylase; BP, benzo[*a*]pyrene.

[2] D. R. Bickers, T. Dutta-Choudhury, and H. Mukhtar, in preparation.

MATERIALS AND METHODS

Chemicals. ^{3}H-Labeled BP 4,5-oxide (specific activity 289 mCi/mmole) and unlabeled BP 4,5-oxide were provided by the Cancer Research Program of the National Cancer Institute, Division of Cancer Cause and Prevention (Bethesda, Md.). The oxides were purified by chromatography on alumina column (as described earlier in refs. 10 and 11) and were >99% pure. Aroclor 1254 was a product of Monsanto (St. Louis, Mo.); Gold Label BP and 7-ethoxycoumarin were obtained from Aldrich Chemical Company (Milwaukee, Wisc.). NADP, NADH, and NADPH were products of Sigma Chemical Company (St. Louis, Mo.). All other chemicals used were of the highest purity commercially available.

Animals. Sperm-positive pregnant Sprague-Dawley rats were obtained from Holtzman Rat Farm (Madison, Wisc.) and shipped during the last trimester. Neonatal rats born *in situ* were allowed to suckle until the 4th day after birth. The animals were withdrawn from their mothers and treated with a single cutaneous application of the polychlorinated biphenyl Aroclor 1254 in 100 μl of acetone (1 mg/10 g) 24 hr before sacrifice. Control animals were treated with acetone alone.

Preparation of tissue. The neonatal rats were killed by decapitation with surgical scissors. The head and extremities of each animal were removed and whole skin from the remaining body, which constitutes 80–90% of total skin, was excised and immediately placed in ice-cold 0.15 M KCl. Each skin was placed epidermal side down on a covered glass Petri dish containing crushed ice. The skin was scraped with a sharp scalpel blade (Bard-Parker No. 20) to remove subcutaneous fat and muscle. For the separation of epidermis and dermis the method of Epstein *et al.* (9) was adapted. Essentially, skins were floated in 0.1 M phosphate buffer (pH 7.40) containing 10 mM dithiothreitol in a 100-ml glass beaker (four skins in each beaker). The beaker was placed on a rotary shaker with gentle shaking in a cold room at 4°. After 2 hr of shaking, the epidermis was peeled away from the dermis using tissue forceps. The whole skin, epidermis, and dermis were washed twice using fresh 0.15 M KCl, blotted with paper towels, and carefully minced to small pieces (<1 mm^3).

Homogenization and subcellular fractionation. All operations were carried out at 4°. The minced tissue was added to a 50-ml polyethylene beaker containing 4 volumes of 0.15 M KCl. Each tissue was subjected to six separate bursts of a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, N.Y.) equipped with an ST-10 generator. There was a pause of 30 sec between each burst to permit cooling of the tissue. This whole homogenate was then poured into the tube of a ground-glass conical tissue homogenizer fitted with a ground-glass pestle made to carefully defined tolerances with a clearance of 0.004–0.006 mm (Kontes Glass Company, Vineland, N. J.) and was homogenized for six passes at 400 r.p.m. using a rotary drill press. This whole homogenate was filtered through two layers of surgical gauze soaked in 0.15 M KCl using gentle vacuum with a Buchner funnel. The homogenate was centrifuged at 800 × *g* for 20 min in a Sorvall RC 2B refrigerated centrifuge using an SS-24 rotor. The pellet was washed gently and recen-

trifuged at 800 × *g* for 20 min. The pooled supernatants were then centrifuged at 9,000 × *g* for 20 min. The 9,000 × *g* pellet was washed and recentrifuged at 9,000 × *g* for 20 min. Unless otherwise stated, this 9,000 × *g* supernatant was used on the day of preparation as the enzyme source for AHH and 7-EC. For spectral analysis and enzyme determinations in microsomes, the pooled 9,000 × *g* supernatants were then centrifuged at 100,000 × *g* for 60 min in a Beckman L 5-50 ultracentrifuge using a 50 Ti-Rotor. The washed microsomal pellet was overlaid with 0.1 M phosphate buffer (pH 7.40) containing 20% glycerol (v/v), 10 mM EDTA, and 10 mM dithiothreitol and was frozen under nitrogen at −170° until the day of analysis (3–5 days after preparation). Verification of the suitability of these procedures for the preparation of skin, epidermal, and dermal microsomes and the stability of the enzyme activity when stored under these conditions will be reported elsewhere.[2]

Spectral analysis. The cytochrome P-450 difference spectra in microsomal preparations obtained from whole skin, epidermis, and dermis were determined spectrophotometrically as described by Omura and Sato (12). Two types of difference spectra were recorded: carbon monoxide plus dithionite-reduced minus carbon monoxide-reduced and carbon monoxide plus dithionite-reduced minus dithionite-reduced. Corrected spectra were obtained using a DW-2a dual beam spectrophotometer (American Instruments Company, Silver Spring, Md.) to which a MIDAN microprocessor in the baseline correction mode was attached. To ensure maximal resolution, all spectral determinations were made at medium response at a rate of 5 nm/sec. An extinction coefficient of 91,000 cm^{-1} m^{-1} was used for the absorbance change between 450 and 490 nm to determine the cytochrome P-450 concentration from the carbon monoxide plus dithionite-reduced minus dithionite-reduced difference spectra. However, for calculation of cytochrome P-450 concentrations from the carbon monoxide plus dithionite-reduced minus carbon monoxide-reduced difference spectra, an extinction coefficient of 100,000 cm^{-1} m^{-1} was used for the absorbance change between 450 and 500 nm. Protein concentrations below 3 mg/ml were used to avoid nonlinear responses.

Enzyme assays. AHH activity was determined by a modification of the method of Nebert and Gelboin (13) as previously described by Mukhtar and Bickers (6). The quantitation of phenolic BP metabolites was based on comparison of fluorescence to a standard solution of 3-OH BP. 7-EC activity was determined according to a slight modification of the procedure of Greenlee and Poland (14). The reaction mixture, in a total volume of 1.0 ml, contained 65 μmole of potassium phosphate buffer (pH 7.2) 1 μmole of NADPH, 1 μmole of NADH, 5.0 μmole of $MgCl_2$, 1 mg of bovine serum albumin, and 2 μmole of 7-ethoxycoumarin (in 50 μl of 50% aqueous methanol). After 30 min of incubation at 37° in air, the reaction was terminated by adding 0.125 ml of 15% (W/V) trichloroacetic acid. 7-Hydroxycoumarin formed during the incubation was extracted into 2 ml of chloroform. A portion (1 ml) of the organic phase was extracted with 1.5 ml of 0.01 N NaOH-1 M NaCl. The amount of 7-hydroxycoumarin formed in comparison with an authentic standard of 7-hydroxycoumarin was determined and expressed as picomoles of product per minute per milligram of protein. Epoxide hydrolase activity in the microsomal fractions was assayed using BP 4,5-oxide as substrate according to the thin-layer chromatographic technique of Jerina *et al.* (15), the details of which were described previously (10, 11).

Protein determination. Protein was determined, after precipitation with trichloroacetic acid, by the procedure of Lowry *et al.* (16) using bovine serum albumin as reference standard.

RESULTS

Histological verification of epidermal/dermal separation. Figure 1A depicts the whole skin of neonatal rats. Figure 1B shows a small segment of typical epidermis obtained by the separation procedure using incubation of skin with 10 mM dithiothreital at 4°. There was clear separation of the epidermis and, most important, no dermal contamination was present. This is a completely reliable separation technique.

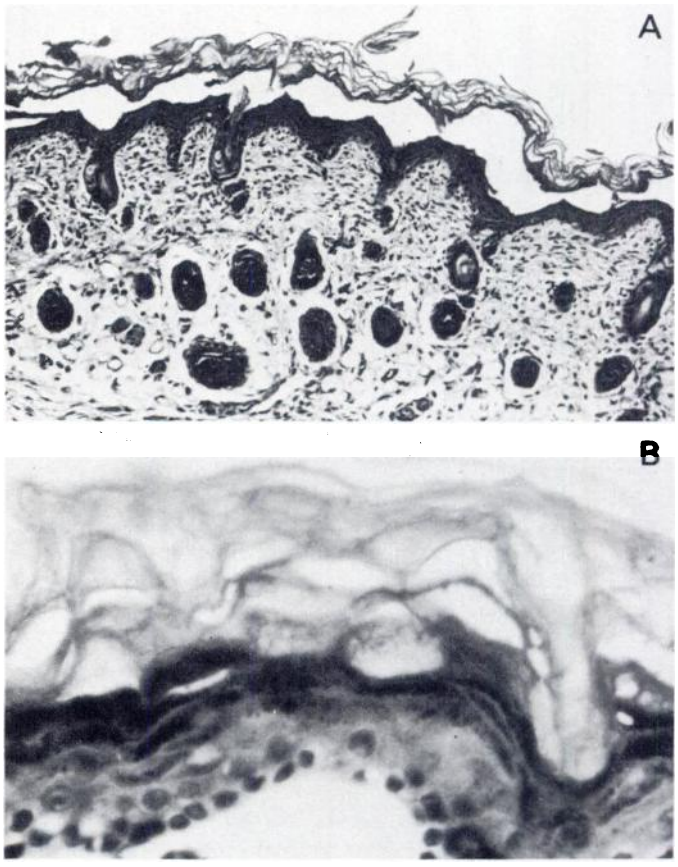

FIG. 1. *Separation of epidermis from whole skin of neonatal rats*

A. Whole skin obtained from a neonatal Sprague-Dawley rat at 4 days of age. The epidermis consists of four or five layers of keratinocytes above which the fibrous stratum corneum can be seen. Beneath the epidermis is the dermis in which numerous hair follicles can be seen (hematoxylin and eosin, ×200).

B. Epidermis obtained after 2 hr of incubation of whole skin in 0.1 M phosphate buffer containing 10 mM dithiothreitol. Note that a clear separation has occurred at the basement membrane (hematoxylin and eosin, ×450.)

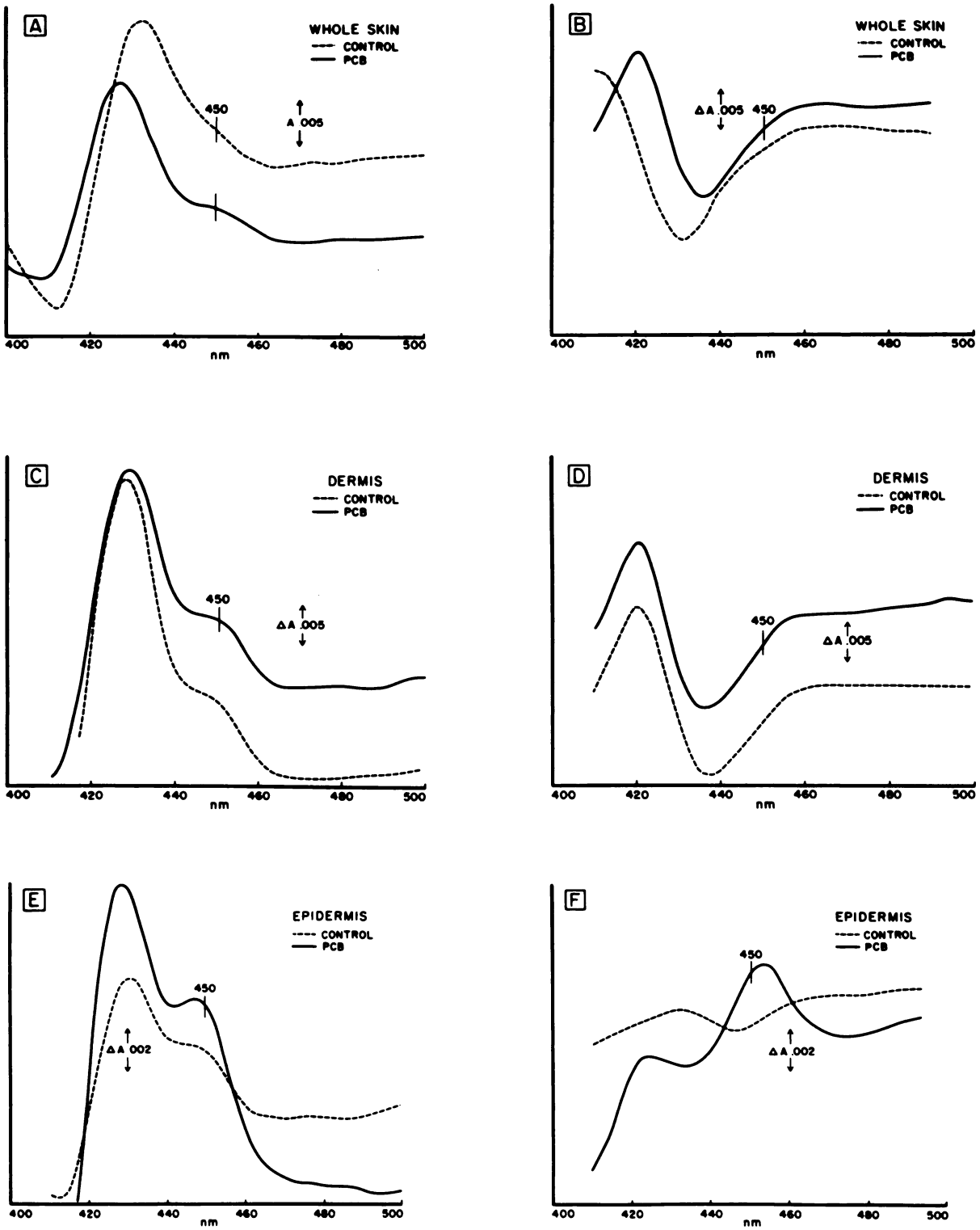

FIG. 2. *Difference spectra obtained from microsomes prepared from whole skin, dermis, and epidermis*

Microsomes from whole skin, dermis, and epidermis were prepared as described under Materials and Methods. CO-dithionite-reduced minus CO-reduced difference spectra are depicted in A, C, and E. CO-dithionite-reduced minus dithionite-reduced difference spectra are depicted in B, D, and F. ——, Aroclor 1254-treated animals; ----- acetone-treated animals. Concentrations of protein (milligrams per milliliter) in the cuvettes used were 2.42, 2.42, 2.34, 2.34, 1.38, and 1.38 for A, B, C, D, E, and F control microsomes, respectively, and 2.65, 2.65, 1.45, 1.45, 2.47, and 2.47 for A, B, C, D, E, and F induced microsomes, respectively.

Cytochrome P-450 levels in microsomes from control and Aroclor 1254-treated whole skin, dermis, and epidermis. Typical and highly reproducible difference spectra were obtained using microsomes from whole skin, dermis, and epidermis of control and Aroclor 1254-treated neonatal rats. These are depicted in Fig. 2. Two types of difference spectra were recorded: (a) carbon monoxide plus dithionite-reduced minus carbon monox-

TABLE 1

Cytochrome P-450 concentrations in microsomes of whole skin, dermis, and epidermis from control and Aroclor 1254-treated neonatal rats

Neonatal rats (4–5 days of age, body weight 9 ± 1 g) were treated with topically applied acetone (control) or with Aroclor 1254 (1 mg/10 gm body weight) in 100 μl of acetone. The animals were killed 24 hr after treatment, and whole skin and liver were removed. Dermis and epidermis from whole skin were separated by flotation of the skin in 0.1 M phosphate buffer (pH 7.40) containing 10 mM dithiothrietol. Microsomes from whole skin, dermis, and epidermis were prepared as described under Materials and Methods. Carbon monoxide plus dithionite-reduced minus carbon monoxide difference spectra were measured as described under Materials and Methods. The protein concentration in the cuvette ranged from 1.36 to 2.64 mg/ml.

Treatment	λ_{max}[a]	Cytochrome P-450	
		pmoles/mg protein	pmoles/gm tissue
	nm		
Whole skin			
Control	452	12.4	30.0
Aroclor 1254	451	14.2	37.6
Dermis			
Control	450	13.7	32.1
Aroclor 1254	450	28.3	71.3
Epidermis			
Control	447	21.7	29.9
Aroclor 1254	446	40.5	100.0
Liver			
Control	452	230.0	943.0
Aroclor 1254	451	482.0	2024.0

[a] The exact wavelength maxima (except those for liver) are best approximates and may vary within 2 nm.

ide-reduced (Fig 2A, C, and E); (b) dithionite plus carbon monoxide-reduced minus dithionite-reduced (Fig 2B, D and F). Because a direct method (CO-dithionite-reduced minus dithionite-reduced) was used, it was not possible to record a difference spectrum from either control or Aroclor-treated whole skin or dermis (Fig. 2B and D), which demonstrated an absorbance peak at or around 450 nm. However, epidermal microsomes from Aroclor 1254-treated rats exhibited a highly reproducible spectrum (Fig. 2F) with absorbance around 450 nm (maximum was between 453 and 454 nm). In epidermal microsomes from control rats an absorbance peak could not be demonstrated. The cytochrome P-450 content of epidermal microsomes, calculated using a molar extinction coefficient of 91,000 cm^{-1} m^{-1} for Aroclor-treated rats, was 17.8 pmoles/mg of protein or 44.0 pmoles/g of tissue. Using an alternate, so-called indirect, method (carbon monoxide plus dithionite-reduced minus carbon monoxide-reduced), it was possible to record difference spectra in microsomes from both control and Aroclor-treated whole skin, dermis, and epidermis. In none of these microsomal spectra could an absorbance maximum be accurately established. However, they did appear to be in the range between 448 and 454 nm. The spectral maxima and cytochrome P-450 concentrations are shown in Table 1. Following topical application of Aroclor 1254, increased amounts of cytochrome P-450 content were seen. The cytochrome P-450 concentrations in epidermal microsomes from control and from Aroclor-induced rats, calculated using a molar extinction coefficientof 100,000 cm^{-1} m^{-1}, were 21.7 and 40.5 pmoles/mg of protein, respectively. The percentage increases observed were 15% for whole skin, 106% for dermis, and 87% for epidermis. It was not possible to determine accurately whether any shift in the wavelength of maximal absorption occurred in microsomes from Aroclor-treated animals following application of the inducer to skin of neonatal rats. This contrasts with data obtained in hepatic microsomes from these animals, in which a 1- to 2-nm shift to the blue was consistently observed (Table 1).

Effect of the topical application of Aroclor 1254 on AHH and 7-EC activity in whole skin, epidermis, and dermis of neonatal rats. To emphasize the differential enzymatic activity in epidermis and dermis, the data in Table 2 are presented in two ways: (a) in picomoles of product per minute per milligram of protein and (b) in picomoles of product per gram of tissue (wet weight). When the data are presented as picomoles of product per minute per milligram of protein there is a 7- to 13-fold enhancement of AHH activity in whole skin, dermis, and epidermis. However, specific activity of both control and induced AHH is highest in epidermis (about 1.5-fold higher than in whole skin or dermis). When the data are presented as picomoles of product per minute per gram of tissue, the higher specific activity of epidermis is further emphasized (2.7 and 1.8 times higher than in whole skin and dermis, respectively). The same pattern

TABLE 2

Effect of topical application of Aroclor 1254 to neonatal rats on AHH and 7-EC activities of whole skin, dermis, and epidermis

Neonatal rats (4–5 days of age; body weight 9 ± 1 g) were treated with topically applied acetone (control) or with Aroclor 1254 (1 mg/10 gm body weight) in 100 μl of acetone. Rats were killed 24 hr after treatment. Whole skin, dermis, and epidermis were prepared as described under Materials and Methods, and enzyme activities were then determined in the 9000 × *g* supernatant fraction. Data represent means ± standard deviation of three individual experiments.

Parameter	Whole skin		Dermis		Epidermis	
	Control	Aroclor 1254	Control	Aroclor 1254	Control	Aroclor 1254
AHH						
pmoles 3-OH BP/min/mg protein	0.51 ± 0.03	3.42 ± 0.09[a]	0.42 ± 0.03	5.59 ± 0.63[a]	0.62 ± 0.05	7.31 ± 0.46[a]
pmoles 3-OH BP/min/gm tissue	9.54 ± 0.63	64.64 ± 1.72[a]	7.06 ± 1.54	94.04 ± 6.32[a]	15.48 ± 1.29	171.50 ± 8.95[a]
7-EC						
pmoles 7-HC[b]/min/mg protein	0.36 ± 0.04	2.84 ± 0.18[a]	0.40 ± 0.03	2.62 ± 0.23[a]	0.49 ± 0.05	2.91 ± 0.20[a]
pmoles 7-HC/min/gm tissue	7.41 ± 0.64	56.97 ± 4.54[a]	6.97 ± 0.54[a]	44.43 ± 3.81[a]	11.96 ± 1.17	71.20 ± 4.63[a]

[a] Statistically different ($p < 0.05$) from corresponding control.

[b] 7-HC, 7-Hydroxycoumarin.

TABLE 3

Effect of topical application of Aroclor 1254 to neonatal rats on microsomal epoxide hydrolase activities of whole skin, dermis, and epidermis

Four-day-old rats were treated topically with either Aroclor 1254 (1 mg/10 gm body weight) or with 100 μl of acetone (control). Rats were killed 24 hr after treatment. Whole skin, dermis, and epidermis microsomes were prepared as described under Materials and Methods, and epoxide hydrolase activities were determined. Data represent means ± standard deviation of three experiments. For treatment and other details see Tables 1 and 2.

EH[a]	Whole skin		Dermis		Epidermis	
	Control	Aroclor 1254	Control	Aroclor 1254	Control	Aroclor 1254
pmoles BP 4,5-diol/min/mg protein	120 ± 6	136 ± 8	135 ± 11	149 ± 15	163 ± 12	198 ± 14[b]
pmoles BP 4,5-diol/min/gm tissue	2185 ± 106	2257 ± 123	2217 ± 176	2506 ± 243	3852 ± 252	4632 ± 312[b]

[a] EH, Epoxide hydrolase (BP 4,5-oxide as substrate).

[b] Significantly different from corresponding control ($p < 0.05$).

of activity was observed with 7-EC as well. These data indicate that the epidermis is the major site of drug metabolism in skin of neonatal rats. To rule out the possibility that the higher activity in epidermis was simply a dose effect whereby the inducer did not adequately penetrate to the dermis, it was observed that i.p. injection of Aroclor 1254 evoked an identical pattern of enzyme induction in whole skin, epidermis, and dermis (data not presented). Furthermore, there was a major increase in hepatic cytochrome P-450 and drug metabolizing enzymes following topical application of these doses of Aroclor 1254 (Table 1).

Effect of topically applied Aroclor 1254 on microsomal epoxide hydrolase. As shown in Table 3, there was measurable activity of epoxide hydrolase in microsomes prepared from whole skin, dermis, and epidermis of control and Aroclor 1254-treated neonatal rats. There was no significant induction of enzyme activity in whole skin or dermis following Aroclor treatment. However, epidermal epoxide hydrolase was significantly induced following topical Aroclor 1254 treatment. The specific activity of epoxide hydrolase was also highest in epidermis compared with dermis and whole skin.

Effect of topically applied Aroclor 1254 on microsomal protein and enzyme activities prepared from liver, whole skin, dermis, and epidermis. As shown in Table 4, the microsomal protein concentrations were similar in whole skin, dermis, and epidermis. Specific activities of AHH and 7-EC in whole skin, dermis, or epidermis were 3.7–5.7% of corresponding hepatic activities. The specific activity of microsomal AHH and 7-EC was highest in epidermis as compared with dermis or whole skin when presented as picomoles of product per minute per milligram of protein and followed the pattern epidermis > dermis > whole skin. In contrast, when presented as total organ activity the epidermal microsomes were least active. However, when total organ activities of AHH or 7-EC of dermis and epidermis were added, approximately whole skin activities were recovered.

Catalytic activities of microsomal cytochrome P-450 of neonatal rat whole skin, dermis, and epidermis and its comparison with hepatic activities. The data in Table 5 compare the capacity of microsomal cytochrome P-450 of control and Aroclor 1254-treated neonatal rat liver, whole skin, dermis, and epidermis to metabolize BP and 7-ethoxycoumarin as measured by the formation of fluorescence products. AHH and 7-EC activities when expressed as product per nanomole of cytochrome P-450 in control whole skin, dermis or epidermis were less than 50% of the hepatic activities. Following topical application of Aroclor 1254 to neonatal rats, the capacity of skin cytochrome P-450 to metabolize these two substrates exceeded that of the corresponding capacity of the hepatic hemeprotein. Furthermore, when the data are expressed as product per nanomole of P-450, whole skin appears to be more active than dermis or epidermis.

DISCUSSION

Skin is one of the body's major direct interfaces with the outside world. Traditional consideration of skin has focused on its structural and functional properties as a relatively impervious barrier between the body and the environment. Skin consists of a thin yet strong structure that contains a number of proteins, among them keratin

TABLE 4

AHH and 7-EC activities in Aroclor 1254-induced whole skin, dermis, and epidermis microsomes

Data represent means ± standard deviation for three experiments. In each experiment, 50 neonatal rats (3–4 days old) were treated topically with Aroclor 1254 (100 mg/kg). Twenty-four hours after the treatment, dermis and epidermis from 40 rats were separated, and whole skin from the remaining 10 rats was used. Microsomes from each pooled tissue were prepared. Total organ activity was calculated by multiplying the specific activity by the total (from one rat) microsomal protein obtained and calculating the mean ± standard deviation for the organ.

	Microsomal protein	AHH		7-EC	
		Specific activity	Total organ activity	Specific activity	Total organ activity
	mg/g tissue	*pmoles/min/mg protein*	*pmoles/min/organ*	*pmoles/min/mg protein*	*pmoles/min/organ*
Whole skin	2.92 ± 0.23	13.3 ± 0.9	27.2 ± 1.8	8.4 ± 0.6	17.2 ± 1.2
Dermis	2.52 ± 0.08	16.4 ± 0.6	20.7 ± 0.8	10.2 ± 0.4	12.9 ± 0.5
Epidermis	3.02 ± 0.09	18.3 ± 0.7	8.8 ± 0.3	12.8 ± 0.9	6.2 ± 0.4
Liver	4.49 ± 0.14	363.2 ± 9.8	412.6 ± 12.3	224.4 ± 12.6	286.3 ± 14.5

TABLE 5

Capacity of microsomal cytochrome P-450 of neonatal rat liver, whole skin, dermis, and epidermis for the metabolism of BP and 7-ethoxycoumarin

Data represent means ± standard deviation for three experiments. In each experiment, 50 neonatal rats (3–4 days old) were treated with either 100 μl of acetone (control) or Aroclor 1254 (100 mg/kg). Twenty-four hours after the treatment, dermis and epidermis from 40 rats were separated, and whole skin and liver from the remaining 10 rats were used. Microsomes from each pooled tissue were prepared, and cytochrome P-450 levels and enzyme activities were then determined.

Tissue	AHH		7-EC	
	Control	Aroclor 1254	Control	Aroclor 1254
	pmoles 3-OH BP/min/nmole P-450		*pmoles 7-HC[a]/min/nmole P-450*	
Whole skin	83 ± 9	937 ± 81	67 ± 8	592 ± 32
Dermis	74 ± 5	580 ± 32	55 ± 6	360 ± 16
Epidermis	59 ± 4	452 ± 18	43 ± 5	316 ± 18
Liver	198 ± 13	754 ± 23	153 ± 12	477 ± 19

[a] 7-HC, 7-Hydroxycoumarin.

and collagen, which provide significant resistance to the damaging effects of physical and chemical agents as well as providing tensile strength for cutaneous tissue. All of these properties have reinforced the general concept that skin is basically inert biologically. However, continuing research has forced a reappraisal of this concept. Mammalian skin contains two major structural components: the outer epidermis and the inner underlying dermis. The epidermis is an actively replicating, compact, stratified squamous epithelium, whereas the dermis has a relatively loose structure consisting of collagen fibers embedded in a ground substance rich in glycosaminoglycans. These different proteins turn over at varying rates, necessitating active protein synthesis.

In recent years it has become clear that the body possesses a system of enzymes that assists in the detoxification of drugs and environmental chemicals (17, 18). This enzyme system was first studied most rigorously in liver and is known as the microsomal mixed-function oxidase or monoxygenase system. It is membrane-bound and requires NADPH and oxygen for catalytic activity. The terminal oxidase of the monooxygenase system is the hemeprotein cytochrome P-450. Virtually all of the early studies of this enzyme system were conducted with the liver, and there is little doubt that hepatic tissue is the major site for drug metabolism in the body.

However, more recent studies over the last decade have revealed that significant drug metabolism occurs in extrahepatic tissues (for a comprehensive update see ref. 19 and chapters therein). Furthermore, it has become clear that the monooxygenase system, although primarily a pathway for the detoxification of exogenous chemicals, may also be capable of metabolizing biologically inert precursors into highly reactive electrophilic species that can bind covalently to cellular macromolecules, thereby evoking a number of toxic effects, among them chemical carcinogenesis (20, 21). Since skin is in direct and continuing contact with numerous potential environmental oncogens (e.g., solar radiation and polycyclic aromatic hydrocarbons) it is essential to develop new knowledge regarding the capacity of skin to metabolize environmental chemicals and to assess the possible consequences of that metabolic activity on toxic responses in cutaneous tissue.

The unique susceptibility of skin to carcinogenesis by environmental chemicals has led to extensive studies of drug metabolism in cutaneous tissue. One enzyme, AHH, appears to play a crucial role in the biotransformation of polycyclic aromatic hydrocarbons into reactive moieties including diol epoxides, which appear to be the ultimate oncogenic metabolites in skin (22–24). The importance of metabolism of polycyclic aromatic hydrocarbons such as BP in inducing cutaneous tumors is clear from studies showing that BP 7,8-dihydrodiol and BP 7,8-diol-9,10 epoxide(s) have tumorigenic effects greater than that of BP itself (25). Suggestions have been made that the responsiveness of AHH to environmental carcinogens correlates with susceptibility to tumorigenesis in experimental animals and in human populations, although the precise relationship between AHH inducibility and susceptibility to chemical carcinogenesis remains controversial (26–29).

In earlier published reports on skin AHH, it was suggested that the specific activity of the enzyme in whole homogenate prepared from isolated mouse epidermis was 4–5 times that found in the dermis and twice that of whole skin (7). In these studies, "epidermal" homogenates were prepared by separation from whole skin using either scalpel scraping or heat treatment (52°). Epidermal homogenates contain large amounts of catalytically inert protein, particularly keratin, and thus could lead to erroneous interpretations if enzyme activities are calculated in terms of whole homogenate protein. In contrast to these findings, Wiebel *et al.* (30) reported that the highest AHH enzyme activity was found in the superficial layer of dermis, which contains sebaceous glands, and the upper pilary canals, which are of epidermal origin embryologically. In these studies it was found that AHH activity was intermediate in the epidermis and was lowest in the deeper dermal layers. This apparent discrepancy may result from epidermal contamination of the tissue preparations used. Among all of the techniques available for separation of dermis and epidermis it had not previously been possible to obtain homogeneous epidermal preparations while retaining catalytic activity for the mixed-function oxidases and epoxide hydrolase. With the technique of Epstein *et al.* (9), epidermal separation is accomplished quickly and simply. We have verified this histologically and have shown that no dermal contamination of these epidermal preparations occurs, and vice versa.

The availability of this technique permitted us to assess the distribution pattern of monooxygenase activities in skin between dermis and epidermis. Previous studies with impure epidermal fractions had been reported only for AHH, and it was not known whether epidermis is a major site for other enzymes, such as 7-EC and epoxide hydrolase as well.

It was not possible to record CO-dithionite-reduced minus dithionite-reduced difference spectra from either control or Aroclor 1254-induced whole skin or dermal microsomes or from control epidermal microsomes. However, epidermal microsomes from Aroclor-treated rats

always exhibited highly reproducible spectra with a maximum between 453 and 454 nm. Since it was not possible to record difference spectra from control epidermal microsomes with confidence using currently available methods, it could not be determined whether any shift in wavelength maxima in Aroclor-treated epidermal microsomes occurred. By using an indirect method in which carbon monoxide is bubbled into both reference and sample cuvettes and the sample cuvette reduced with dithionite, difference spectra were obtained in microsomes prepared from either control or treated whole skin, dermal, or epidermal microsomes. Whole skin exhibited only a 15% increase in cytochrome P-450 concentrations, whereas approximate doubling of values occurred in dermis and epidermis. This differential response observed in cytochrome P-450 inducibility following Aroclor application to neonatal rat skin may be due to removal of the contaminating peak around 430 nm, which represents the major absorbing chromophore in these microsomes. No attempt was made in this study to determine the nature of this peak. Since the microsomal preparations were washed thoroughly and since epidermis is avascular, hemoglobin (418 nm) contamination is unlikely. This absorption peak might represent cytochrome b_5 (423 nm). However, the possibilities of degradation of cytochrome P-450 to cytochrome P-420 and contamination with other heme pigments cannot be excluded. It is clear from these studies that much additional work needs to be done to better characterize cytochrome P-450 in skin. The precise relationship between this microsomal hemeprotein and associated monooxygenase enzyme activities in skin remains to be defined. Efforts to solubilize and purify this microsomal hemeprotein are a major continuing effort of our laboratory.

AHH and 7-EC were found to be induced in neonatal rat skin by topical application of Aroclor 1254. The extent of induction in whole skin, dermis, and epidermis was comparable. Specific activities expressed in terms of product per milligram of protein or per gram of tissue were much higher (1.5–2.8 times) in epidermis of control or induced rats as compared with whole skin or dermis. Induction of epoxide hydrolase was apparent only in epidermis. To our knowledge, this is the first report showing that epoxide hydrolase activity can be induced in epidermis. It should be emphasized that the induction, although small, was statistically significant. The role of this enzyme in epidermis deserves further study as well.

Although skin cytochrome P-450 levels in neonatal rats are only a fraction of those present in liver (Table 1), skin cytochrome P-450 of Aroclor 1254-treated animals nonetheless appears to metabolize BP and 7-ethoxycoumarin at least to the extent achieved by the liver cytochrome. In untreated animals, whole skin, dermal, or epidermal microsomal cytochrome P-450 has less than one-half the capacity of liver to metabolize these two substrates (Table 5). This is consistent with our recent study (6) in which it was found that, after topical application of Aroclor 1254 to neonatal rats, skin AHH and 7-EC increase to a greater extent than do the liver enzymes.

In summary, the results presented in this paper have shown for the first time that pure epidermal microsomes contain inducible AHH, 7-EC, and epoxide hydrolase activity as well as spectrally detectable amounts of the hemeprotein cytochrome P-450. Our data re-emphasize that the epidermis is an important site of xenobiotic metabolism in skin of neonatal rats. Further studies are in progress to better define the range of substrates metabolized by the skin monooxygenase system.

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