Intact, viable (>80%) epidermal cells were isolated from the hairless mouse. These cells metabolized 7-ethoxycoumarin (7-EC) to umbelliferone (UMB) (3 pmol/min/10^6 cells) and UMB to the sulfate and glucuronide conjugates (1 pmol/min/10^6 cells). The rate of oxidation in intact cells compared well with that in disrupted cells with added NADPH, but conjugation proceeded more rapidly in disrupted cells with added cofactors due to a combination of "activation" of the UDP-glucuronosyltransferase, and to a limitation of activity by the concentration of UDP-glucuronic acid in the intact cells. Pretreatment of the animals with 5,6-benzoflavone resulted in a 5-fold increase in the rate of oxidation, and a 2-fold increase in both the rate of conjugation and the intracellular concentration of UDP-glucuronic acid. UDP-glucuronic acid concentration in isolated increased during incubation with glucose, and was regenerated to a steady-state concentration on incubation of cells with UMB. Pretreatment of animals with 5,6-benzoflavone decreased the percentage of metabolite conjugated (from 30% to 15%), whereas adding an inhibitor of oxidation, ellipticine, to cells isolated from pretreated animals, increased the percentage of metabolite conjugated (from 15% to 40%). Sulfation of UMB was almost undetectable, except at very low concentrations (<10 nM) of substrate. Thus, glucuronidation of UMB in epidermal cells may be limited by UDP-glucuronic acid availability; sulfation in the epidermis may contribute little to the conjugation of UMB; and >70% of the products of 7-EC oxidation in the skin may remain unconjugated.

Metabolism of drugs and other foreign compounds often occurs by more than one sequential reaction. The initial reaction is frequently oxidative, and is often catalyzed by enzymes of the cytochrome P-450 family. The products of this reaction may then be further metabolized, e.g., by glucuronidation or sulfation. The first of the two stages, oxidation, has been demonstrated by several laboratories to occur in the skin [1-7]. This activity is predominantly located in the epidermis. Conjugation reactions in the skin have not been examined as extensively. Aitio and Marniemi [8] mention four publications concerning glucuronidation in skin strips or homogenized skin, and there are two more recent reports of glucuronidation by skin microsomes [6,9]. Powell and Roy [10], in a review on extrahepatic sulfation, make no reference to this activity in skin, while Harper and Calcutt [11] and Moloney et al. [12] report finding only low levels.

Since the product of the oxidative reaction serves in many instances as the substrate for conjugation, it is important to examine these two reactions in a system that is as close as possible to intact tissue. This has been done in other organs, such as liver, by using either the perfused organ [13-15] or isolated hepatocytes [16-18]. In skin, perfusion is an imprecise technique and thus the experimental system of choice is the intact cell. Our laboratory has developed a method for preparing epidermal cells that are greater than 80% viable [19] and this paper describes the two stages of the metabolism of 7-ethoxycoumarin in these epidermal cells.

**MATERIALS AND METHODS**

**Preparation of Cells**

Epidermal cells were isolated from female hairless mice (HRS/J, aged 7–10 weeks, purchased from Jackson Laboratories, Bar Harbor, Maine) by incubation with nonspecific protease from Streptomyces griseus (prase obtained from Boehringer-Mannheim, Indianapolis, Indiana) as described previously [19]. Cells were counted and viability ascertained after mixing with ethidium bromide and fluorescein diacetate and were disrupted by sonication when required [19]. Some mice were pretreated by topical application on the back with 1.25 mg 5,6-benzoflavone in 100 µl acetone 24 h before killing.

**Metabolism of 7-Ethoxycoumarin and Umbelliferone**

Oxidation of 7-ethoxycoumarin (7-EC) to 7-hydroxycoumarin (umbelliferone, UMB), and glucuronide and sulfate conjugation of UMB were measured as follows: cells (0.5–5 x 10^6/ml) were incubated at 37°C in HEPES buffered solution (HEPES), pH 7.4 (containing 150 mM NaCl, 5 mM KCl, 4 mM KHPO₄, 5.5 mM glucose, and 25 mM HEPES), with added (0.04%) deoxyribonuclease from bovine pancreas (Sigma Chemical Co., St. Louis, Missouri), 7-EC, synthesized according to Ullrich and Weber [20], was dissolved in 0.1 M HEPES buffer, pH 7.6, and added to the cells to the desired concentration. Reactions were terminated by the addition of 5 ml ethyl ether containing 1.5% isoamyl alcohol (ether/IAA) to 1 ml of the reaction mixture and the unconjugated (free) UMB was extracted into the organic phase. Two milliliters of this organic phase was then extracted with 3 ml of 0.2 M glycine-NaOH, pH 10.4. The free UMB can be measured in the aqueous phase by reading fluorescence (excitation 375 nm; emission 458 nm). The aqueous phase remaining after ether extraction of the incubation mixture contained conjugated UMB. To this was added 0.5 ml of limpet β-glucuronidase, 1000 units/ml, in 0.2 M acetate, pH 4.5 (Sigma Chemical Co.), which also has sulfatase activity. Incubation for 30 min at 37°C resulted in quantitative hydrolysis of conjugated UMB. This free UMB was extracted into ether/IAA and then into glycine for fluorescence measurement as described above. When UMB was used as substrate, the free UMB was extracted into ether/IAA and discarded; the conjugated UMB was measured as described above. UMB standards were carried through the extraction procedure as appropriate; recovery was > 99%.

To differentiate between sulfate and glucuronide conjugates, duplicate 1-ml samples of conjugated UMB were incubated with each 10 units sulfatase Type H1 from Helix pomatia (Sigma Chemical Co.) added in 0.5 ml 0.2 M acetate, pH 4.5, together with 20 mM saccharo-
1,4-lactone (Calbiochem-Behring, San Diego, California) to inhibit β-glucuronidase, or with β-glucuronidase as described above. The incubation with sulfatase liberated UMB from sulfoconjugates, and the β-glucuronidase treatment hydrolysed both glucuroni- and sulfate-conjugated UMB. Uridine-5′-diphosphogluconic acid (UDPGA) and adenosine 3′-phosphate 5′-phosphosulfate (PAPS) were both purchased from Sigma Chemical Co.

**Measurement of UDPGA**

UDPGA was measured by a modification of the method of Wong and Sorous [21]. Cells or tissue were boiled in HBPS for 5 min, centrifuged, and the supernatant used as a source of UDPGA for the conjugation of UMB or 4-methylumbelliferrone (4-MU). Incubation mixtures of 0.9 ml contained 0.02 units rabbit liver UDP-glucuronosyltransferase Type II (Sigma Chemical Co.) and 25 nmol UMB or 20 nmol 4-MU and 0.3 ml of UDPGA standards or supernatant; this was incubated for 30 min at 37°C. The incubation mixtures containing UMB were treated as described above to measure conjugated UMB. The incubations with 4-MU were terminated by adding 2.5 ml 0.05 M sodium acetate, pH 4.5, and excess 4-MU was extracted into a total of 45 ml chloroform by 3 extractions. One milliliter of the aqueous phase was added to 1 ml 0.01 M sodium acetate, pH 4.5, and treated with β-glucuronidase as described previously. Glycine (1.5 ml, 0.2 M, pH 10.4) was added and fluorescence of 4-MU determined (excitation 370 nm/emission 450 nm).

**NADPH Generating System**

When required, NADPH was generated by adding (to a final concentration) 5 mM glucose-6-phosphate, 5 mM MgSO₄, 0.67 NADPH, and 1 unit/ml glucose-6-phosphate dehydrogenase from Torula yeast, Type XI (Sigma Chemical Co.).

**RESULTS**

**Metabolism of 7-EC and UMB by Epidermal Cells**

The skin has been reported to have significant β-glucuronidase activity [22]. After disruption of the cells and acidification (needed for optimal glucuronidase activity) of the medium, hydrolysis occurred at a measurable rate. To ascertain whether this reaction would affect measurement of the rate of conjugation in intact cells, the disappearance of UMB was measured in intact cells in the presence of 3 mM saccharo-1,4-lactone, which inhibits β-glucuronidase (in skin homogenate) completely at this concentration. Under these conditions there was no difference in disappearance of the fluorescent UMB (with or without saccharo-1,4-lactone), which indicated that the conjugated UMB was not being hydrolyzed back to free UMB, and it was concluded that in the intact cell β-glucuronidase activity would not affect measurement of conjugation rates. Moloney et al. [12] found no sulfatase activity in skin strips. Isolated intact epidermal cells from the hairless mouse metabolized UMB to the glucuronide and sulfate conjugates of this compound at a rate that was linear for 30 min. Pretreatment of the mice by topical application of 5,6-benzoflavone increased this rate from 1 pmol/min/10⁶ cells to > 2 pmol/min/10⁶ cells. The concentration of O-4 MUM (5 μM) in these incubations was below saturation (unpublished results) for this system, and is within the concentration range which may be calculated to result from O-dealkylation of 7-EC by these cells, when the cells are suspended at 5 x 10⁶/ml.

The intact cells O-dealkylated 7-EC, and pretreatment with 5,6-benzoflavone increased this activity 5-fold, from 3 pmol/min/10⁶ cells to greater than 15 pmol/min/10⁶ cells. The concentration of 7-EC used (200 μM) was near the limit of solubility for 7-EC in aqueous buffer and was chosen as consistent with earlier experiments where metabolism by microsomes or disrupted cells was measured [2,7]. In cells from control animals, the ratio of free UMB to conjugated UMB after a 30-min incubation was 2:1 (Fig 1A). Since pretreatment of animals with 5,6-benzoflavone increased the activity of the mixed-function oxidase (cytochrome P-450) more than that of the conjugating enzymes (UDP-glucuronosyltransferase and sulfotransferase), the ratio of free to conjugated UMB in cells from pretreated animals changed to 4:1 over this time period (Fig 1B).

**Effect of Inhibitors on 7-EC and UMB Metabolism**

The ratio of free to conjugated UMB, as well as the rates of oxidative deethylation and conjugation could also be altered including inhibitors in the incubations. Ellipticine, a potent inhibitor of 7-ethoxycoumarin O-dealkylation [23], inhibited this reaction 100% in cells isolated from untreated animals. Thus cells from 5,6-benzoflavone-pretreated animals (90% inhibition of O-dealkylation) were used to show the effect on conjugation of inhibition of oxidation of 7-EC. The Kₘ (UMB) for UDP-glucuronosyltransferase in hairless mouse epidermal cells was determined experimentally to be 7 μM. Since the concentration of UMB produced by the action of the mixed-function oxidase was below this concentration, this decrease in the rate of oxidation should lead to a decrease in the rate of conjugation, and this is shown in Table I. Ellipticine was shown not to affect the rate of the conjugation reaction itself.

Saliclyamide is a competitive substrate for both glucuronidation and sulfation [24]. In skin cells, this compound affected neither the rate of 7-ethoxy­coumarin O-dealkylation nor the rate of conjugation of UMB (Table I).

**Metabolism of 7-EC and UMB by Sonicated Cells**

The two stages of 7-EC metabolism appeared to be independent of each other and this was confirmed by further experiments. Epidermal cells were disrupted by sonication, NADPH was provided by a regenerating system, and 7-EC metabolism was measured in the presence and absence of added

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**FIG 1.** Metabolism of 7-EC to free and conjugated UMB in intact cells. Cells were incubated with 200 μM 7-EC. A. Cells from untreated animals. B. Cells from animals pretreated with 5,6-benzoflavone. Values are the means of 3 experiments ± SD. (○) free UMB; (□) conjugated UMB.

**TABLE 1.** Effect of inhibitors on 7-EC metabolism by skin cells from control or 5,6-benzoflavone-pretreated mice

<table>
<thead>
<tr>
<th>Treatment/additions</th>
<th>Free UMB*</th>
<th>Conjugated UMB*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,6-Benzoflavone pretreated</td>
<td>19.0</td>
<td>2.5</td>
</tr>
<tr>
<td>5,6-Benzoflavone pretreated + 1.25 μM ellipticine</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Untreated</td>
<td>1.7</td>
<td>0.25</td>
</tr>
<tr>
<td>Untreated + 500 μM salicylaldehyde</td>
<td>2.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* pmol/min/10⁶ cells; 20-min incubation.

Ellipticine is first dissolved in DMSO and then diluted with HEPES buffer for addition to incubation mixtures. DMSO alone at the final concentration used has no effect on 7-EC metabolism or UMB conjugation.
UDPGA. The total metabolism of 7-EC remained approximately the same, even though in the absence of added UDPGA no conjugates were formed.

Glucuronidation and Sulfation of UMB

UMB is conjugated by both UDP-glucuronosyltransferase and sulfotransferase. In other systems, it has been shown that the relative amounts of the two conjugates depend on substrate concentration [16,17]. Fig 2A, B show the results of experiments that employed different incubation conditions. With 5 μM UMB as substrate, the sulfated product was barely detectable (data not shown). To generate UMB at a low but self-renewing concentration, 7-EC was used as the starting substrate. At 5 min the sulfated conjugate was of nearly equal concentration with the glucuronide (Fig 2A) and this suggested that even earlier time points should be examined. The results (Fig 2B) show that the lower UMB concentrations at these early times (approximately 10 nM at 5 min under the conditions of these experiments) support sulfation as well as glucuronidation.

Effect of UDPGA Concentration on Glucuronidation Rate

After sonication, cells often exhibited higher rates of conjugation of added UMB than was seen in intact cells; increasing the UDPGA concentration from 0.5 mM to 2 mM further increased these rates. For example, whole cells with 10 μM UMB formed 1-4 pmol UMB-glucuronic acid/min/10^6 cells while sonicated cells with 10 μM UMB and 1 mM UDPGA formed 4-7 pmol UMB-glucuronic acid/min/10^6 cells (range of 3 experiments). These results were difficult to interpret, since UDP-glucuronosyltransferase exhibits “latency” and may have been activated by sonication [25]. Several approaches were used to determine whether this stimulation in the skin cells by added UDPGA was indicative of limiting intracellular UDPGA. It was noted that the amount of stimulation by added UDPGA (> 100%) was greater than the fraction of the cells that were nonviable (< 20%) and thus expected to respond to exogenous cofactor. This would be consistent with a limitation of activity by endogenous cofactor. Secondly, UDPGA concentration was measured directly (Table II). Pretreatment of the mice with 5,6-benzoflavone increased the concentration of UDPGA in the cells; during a 30-min incubation of cells in HBPS the concentration of UDPGA rose to twice the starting value. The ability of the isolated cells to synthesize UDPGA was also shown by incubating cells with UMB, and then measuring the amount of UDPGA remaining. In 30 min 26 pmol of UDPGA was used by 10^6 cells to conjugate the UMB, but the amount of free UDPGA was the same as that in cells that had been incubated without UMB.

Additional glucose (20 mM) was added to the incubation medium, but no increase in conjugation was found, suggesting that UDPGA synthesis was maximal in the incubations which routinely contained 5.5 mM glucose. NAD+ is required for the synthesis of UDPGA, and when 5 μM antimycin A or 1 μM rotenone was added to the incubation to prevent reoxidation of NADH, both UDPGA concentration and the rate of conjugation decreased (e.g., with antimycin A, from 38 to 16 pmol UDPGA/10^6 cells and from 1 to < 0.1 pmol UMB conjugated/mn/10^6 cells). This suggested that the rate of conjugation was indeed limited by UDPGA availability.

Effect of PAPS Concentration on Sulfation Rate

Although the rate of conjugation of UMB with UDPGA may be limited by cofactor, this does not seem to be the case for sulfation. Addition of cysteine (4 mM) and sulfate (0.5 mM) to the incubation medium, which normally did not contain either of these compounds, did not alter the rate of sulfation. When cells were disrupted and incubated with 5 μM UMB and 0.5 mM PAPS, sulfation was not stimulated as compared with intact cells.

Table II. Effects of incubation with and without UMB on the UDPGA content of skin cells from control or 5,6-benzoflavone-pretreated mice

<table>
<thead>
<tr>
<th>Treatment/addition</th>
<th>pmol UDPGA/10^6 cells a</th>
<th>0 Min b</th>
<th>30 Min b</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,6-Benzoflavone pretreated</td>
<td>34 (29, 38)</td>
<td>79 (75, 83)</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>20 (21, 14, 25)</td>
<td>35 (35, 40)</td>
<td></td>
</tr>
<tr>
<td>Untreated + 5 μM UMB</td>
<td>20 (21, 14, 25)</td>
<td>32 (18, 40)</td>
<td></td>
</tr>
</tbody>
</table>

a Average (individual values).

b UDPGA was measured immediately following isolation of cells and after the cells had been incubated in HBPS at 37°C for 30 min.

Additional results are shown in Fig 1. The presence of an inhibitor of oxidation also altered the ratio (up to 40% was

DISCUSSION

Oxidation of 7-EC in the intact epidermal cells (3 pmol/min/10^6) compared favorably with metabolism by cells disrupted by sonication and supplemented with cofactor [7,9]. Maloney et al [12] measured 7-EC O-deethylation by skin strips from the hairless mouse. The area of skin that yielded 100 mg dry weight as reported by these authors (9-10 cm^2), in our hands yielded approximately 20 million cells, and the rates in the two systems compare well (2 pmol/min/5 mg dry weight in skin strips vs 3 pmol/min/10^6 cells). The conjugation rate we have reported here for cells seems, however, much lower than that reported by Maloney et al [12] using skin strips (1 pmol/min/10^6 cells vs 22 pmol/min/5 mg dry weight). This may, in part, be due to a difference in substrate concentration (5 μM vs 70 μM), or to difference in activation of the transferase enzymes, or to animal strain.

It has been shown in several laboratories including our own that cytochrome P-450-dependent enzyme activity in the skin can be increased by topical application of inducers [2,3,7]. Our laboratory has also shown that conjugation with UDPGA in disrupted skin cells may be similarly affected [7]. Moloney et al [6] have shown that 3-methylcholanthrene administered i.p. can double glucuronidation rates of 1-naphthol by skin microsomes but that UMB glucuronidation in skin strips was not affected. The results in this paper indicate that there was a differential increase in the rates of the two stages of metabolism of 7-EC in intact skin cells after topical application of 5,6-benzoflavone and that this resulted in a change in the ratio of free to conjugated metabolites, from 15% conjugated in control animals to 3% in treated animals (Fig 1). The presence of an inhibitor of oxidation also altered the ratio (up to 40% was
conjugated when oxidation was inhibited in cells from pre-treated animals).

Both sulfation and glucuronidation of UMB could be measured in the epidermal cells, but sulfation rates were low (Fig 2). Harper and Calcutt [11] reported finding only a "trace" of sulfation by skin strips and we found no other reports of sulfation in epidermis. Many substrates seem to be preferentially glucuronidated or sulfated in intact cells or perfused organs at high or low substrate concentration, since sulfotransferase has a lower Km but also lower Vmax than UDP-glucuronosyltransferase in many tissues [16,17,26,27]. To generate UMB at a low but steady rate, 7-EC was incubated with cells from untreated animals. Only a very slight increase in sulfation could be measured, suggesting that the low sulfation rate in mouse epidermis is a function of limiting enzyme. The other possibility, that PAPS was limiting, appears unlikely, since disrupted cells with added PAPS showed only limited sulfation and addition of 4 mM cysteine and 50 μM sulfate to the incubation medium, which was shown by Schwartz [18] to enhance sulfation in hepatocytes, had no effect on sulfation by epidermal cells. It would appear that in mouse skin, as has been suggested for mouse generally, sulfation plays little part in conjugation.

Although glucuronidation appeared to be the predominant pathway for UMB conjugation in the skin, most of the product of 7-EC metabolism remained unconjugated (Fig 1). Since formation of UDPGA is energy dependent, it seemed likely that the skin, a relatively energy-poor tissue, might have limiting UDPGA. In the whole skin of the hairless mouse there was 80 nmol UDPGA/g tissue. Epidermal cells had 20–30 pmol UDPGA/10⁶ cells (Table II). It appears that the UDPGA pool in the cells can be maintained during conjugation over a 30-min period and, in fact, increases (Table II). That the rate of conjugation is determined, in part, by availability of UDPGA, was also shown by the effect of topical treatment of animals with 5,6-benzoflavone, which increased both UDPGA concentration and the conjugation rate (Table II); and by preincubation with antimycin A or rotenone which reduced both. This inductive effect of 5,6-benzoflavone on UDPGA concentration and conjugation rate has also been shown in the liver [13].

It has been shown that UDP-glucuronosyltransferase is activated by a variety of treatments [25] and we have shown damaged skin cells indeed exhibited higher rates of conjugation of UMB than intact cells. Moloney et al [6] also report the activation of this enzyme in microsomes from skin.

The experiments outlined in this paper indicate that the major pathway for conjugation of UMB in isolated skin cells depends on UMB concentration, but that the majority of the UMB formed from 7-EC by such cells remains unconjugated. The conjugation that does occur is sensitive to circumstances which alter the availability of UDPGA. These results also exemplify the usefulness of the intact, viable epidermal cell preparation developed in our laboratory [19].

REFERENCES


