

In Vitro Absorption of Some o-Phthalate Diesters Through Human and Rat Skin

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The absorption of undiluted phthalate diesters [dimethyl phthalate (DMP), diethylphthalate (DEP), dibutyl phthalate (DBP) and di-(2-ethylhexyl)phthalate (DEHP)] has been measured *in vitro* through human and rat epidermal membranes. Epidermal membranes were set up in glass diffusion cells and their permeability to tritiated water measured to establish the integrity of the skin before the phthalate esters were applied to the epidermal surface. Absorption rates for each phthalate ester were determined and a second tritiated water permeability assessment made to quantify any irreversible alterations in barrier function due to contact with the esters. Rat skin was consistently more permeable to phthalate esters than the human skin. As the esters became more lipophilic and less hydrophilic, the rate of absorption was reduced. Contact with the esters caused little change in the barrier properties of human skin, but caused marked increases in the permeability to water of rat skin. Although differences were noted between species, the absolute rates of absorption measured indicate that the phthalate esters are slowly absorbed through both human and rat skin.

Introduction

Plastics and materials containing plastics are ubiquitous in modern society. Some plastic is rather inflexible and brittle, so adjuvants are added to the polymerized plastic matrix to enhance flexibility and add color. Among the most common adjuvants are the *o*-esters of phthalic acid, which may make up to 50% by weight of the product (1). They are not irreversibly bound in the plastic matrix and so are free to diffuse. Human exposure to these diffusible adjuvants is, therefore, of toxicological interest.

Following the reports that mice and rats fed di-(2-ethylhexyl)phthalate (DEHP) in their diet developed tumors (2,3), the safety and toxicity potential to exposed humans has been of some concern. Such esters of phthalic acid are lipophilic, and it has been suggested that absorption through skin would be a likely route of human exposure (4). Compared to the fairly comprehensive data available on absorption of phthalate esters following oral administration of the esters, there is an absence of data on percutaneous absorption.

We have measured the percutaneous absorption of a series of typical phthalate esters through human and rat epidermal membranes mounted in glass diffusion cells. In this *in vitro* technique, absorption is directly through the epidermis, whose outermost layer, the stratum corneum, is the principal barrier to diffusion (5).

The esters were applied directly to the epidermal membranes, i.e., as the neat liquids, and not formulated as an excipient in another vehicle.

Materials and Methods

Materials

The phthalate diesters used in this study and their physicochemical properties are listed in Table 1. The diesters were all commercially available compounds (Aldrich Chemical Co. Ltd., Gillingham, Dorset, UK) with a stated purity of 99%. [¹⁴C]DEHP was supplied by Amersham International PLC (Buckinghamshire, UK) and had a purity >99%. The [¹⁴C]DEHP was added to unlabeled DEHP to give a final specific activity of approximately 35 μ Ci/mL. Tritiated water (Amersham International) was diluted with distilled water to a final activity of 5 μ Ci/mL.

Analytical Techniques

The amount of DMP and DEP in samples was determined with a Pye Unicam SP-100 spectrophotometer, using a scanning mode in the range 350 to 220 nm with measurements made at peak absorbance. The molar extinction values (ϵ) of DMP and DEP were 1.31 and 1.30, respectively, at a wavelength of 275 nm in 50% v/v aqueous ethanol. DMP, and some DEP samples, were analyzed using a gas chromatographic technique employing a flame ionization detector set at 300°C. The

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instrument used was a Hewlett-Packard 5736A chromatograph fitted with a Hewlett-Packard 3390A integrator and Hewlett-Packard 7672A automatic sampler. The 90 cm \times 4 mm ID glass column was packed with 4.1% OV 210 on Supelcort (100–120 mesh). Column temperature was 160°C for DEP and 180°C for DBP. The carrier gas used was nitrogen at a flow rate of 60 mL/min.

All radiochemical assays were done using a liquid scintillation counter (model BETAmatic II, Kontron Instruments, Welwyn Garden City, UK). Samples were placed in Optiphase MP (10 mL; Fison's Ltd., Bakewell Road, Loughborough, Leicestershire, UK). Constant counting efficiency was obtained in this scintillation medium.

Preparation of Epidermal Membranes

Human abdominal skin was obtained from cadavers. The donors were mostly female and 55 years old or more. The subcutaneous fat was removed and the skin immersed in water at 60°C for 40 to 45 sec. The epidermis was peeled away from the dermis and the epidermal sheet floated onto water and then taken up onto aluminium foil. The epidermal membranes were stored at 4°C until required (within 7 days of preparation). Such stored membranes have been shown to maintain their permeability properties (6).

Rats (AL/pk, Wistar-derived strain, Animal Breeding Unit, Alderley Park, Macclesfield, Cheshire, UK) were humanely killed by overexposure to Fluothane (Halothane BP; ICI PLC, Alderley Park, Macclesfield, Cheshire, UK) followed by cervical dislocation. The dorsal skin was removed and placed in 2 M NaBr for up to 24 hr (7). After blotting the epidermis dry, it was peeled from the dermis and stored on aluminium foil at 4°C until required (as with the human epidermis, within 7 days of preparation).

Two different methods have been used for the preparation of the human and rat epidermal membranes. Human epidermal membranes can be prepared by either heat or NaBr separation. The membranes produced have the same permeability properties independent of the preparation technique (8).

Skin Absorption Measurements

The epidermal membranes on aluminum foil were cut into discs, 3 cm in diameter, floated onto water, and drawn over the receptor chamber of a glass diffusion cell (9). The integrity of all membranes was assessed by measuring the permeability to tritiated water on day 1 of the experiment. The permeability was expressed as a permeability constant (K_p , units cm/hr) and human membranes with values greater than 1.5×10^{-3} cm/hr and rat membranes greater than 2.5×10^{-3} cm/hr were considered to have an altered epidermal diffusion barrier and were rejected. Tritiated water was desorbed from the membranes in to saline at the end of the experiment (6 hr contact time). Overnight (15 hr) the do-

nor chamber was left empty and 0.5 mL saline was put in the receptor chamber (volume 4.0–5.0 mL). This saline maintained a high relative humidity in the receptor chamber. Thus, the membrane was allowed to hydrate naturally overnight.

On day 2 of the experiment the receptor chambers (approximate volume, 4.5 mL) of the diffusion cells were filled with a known volume of 50% v/v aqueous ethanol and approximately 0.5 mL of a phthalate ester was applied onto the epidermal surface in the donor chamber, ensuring that the entire surface of the membrane was covered. Samples (0.5 mL for DMP, DBP, and DEP; 50 μ L for [14 C]DEHP) were taken frequently during the course of the experiments from the receptor chamber and analyzed for the test phthalate ester as described above. Samples were replaced with an equal volume of fresh receptor fluid. During all experiments the diffusion cells were maintained at $30 \pm 1^\circ\text{C}$.

The length of the experiment varied with the skin type and compound. Studies using rat epidermis were satisfactorily concluded within 8 hr, although the [14 C]DEHP experiments were continued for up to 53 hr. Human skin experiments were continued for 30 hr, except, again, for the [14 C]DEHP experiment, which continued for 72 hr.

The steady-state rate of absorption (units, $\mu\text{g}/\text{cm}^2/\text{hr}$) for each compound was calculated from the linear portion of a plot of cumulative amount of ester penetrated versus time. Permeability constants were calculated as reported previously (9).

At the end of the experiment, the ester was carefully washed off the donor surface, the receptor chamber was emptied, and a second determination of the permeability of each membrane to tritiated water was then done as described for day 1 of the experiment. By dividing the second tritiated water permeability result by the first result, a damage ratio was calculated. This is an indication of an irreversible alteration in the barrier properties of the epidermal membrane caused by contact with the phthalate ester.

Determination of Physicochemical Characteristics

Most of the physicochemical values required were already available, but some were generated afresh as a check on the specific batches of purchased chemicals.

Molecular Dimensions (Volume). The molecular volume of each of the esters was determined by constructing scale models using CPK Atomic Models (CPK, Ealing, UK), covering the models with a plastic film coating, and then displacing water in a beaker.

Water Solubility. 0.5 mL of the ester was shaken with 8 mL distilled water and the resultant suspension centrifuged (Heraeus Labofuge 6000, Heraeus-Christ, GmbH, Osterode) at 2000 rpm for 30 min. An extract of the aqueous phase was further centrifuged (same conditions) for 30 min. A sample was then analyzed (Pye-Unicam SP8-100 spectrophotometer) for ester content.

Octanol/Water Partition Coefficient (Lipophilicity). The phthalate ester was dissolved in 10 mL of octanol and then added to 10 mL water (both the octanol and water were previously saturated with one another). The mixtures were shaken for 30 min and then centrifuged for 2 hr at 2000 rpm (Beckmann model TJ-6 centrifuge Beckmann, Buckinghamshire, UK). An aliquot was taken from the aqueous phase and further centrifuged before the ester content was assayed spectrophotometrically and the octanol/water partition coefficient ($P_{\text{oct/water}}$) calculated.

$$P_{\text{oct/water}} = \frac{\text{concentration of phthalate in octanol}}{\text{concentration of phthalate in water}}$$

Similarly, the $\log_{10}P$ value was calculated thus;

$$\log_{10}P = \frac{\log_{10}P \text{ concentration of ththalate in octanol}}{\log_{10}P \text{ concentration of phthalate in water}}$$

Results

Following applications to the skin, a lag phase followed by a linear phase of absorption was detected for each phthalate diester. From the linear phase of the absorption versus time curve, steady-state absorption rates were determined for each of the phthalate diesters through both human and rat skin (Table 1). Human skin was less permeable than the rat skin for all four diesters. Extrapolation of the linear phase allowed a lag time to be defined for each ester (Table 1). Generally, there was a trend to an increasing lag time with increasing molecular weight, but this relationship was not absolute.

The esters had similar molecular volumes (Table 2) with a range of less than a factor of two. Overall, the diesters showed a 300-fold range of aqueous solubility and a wide range of lipophilicity (Table 2). Absorption rate has been compared with lipophilicity (by $\log_{10}P$) (Fig. 1) and aqueous solubility (Fig. 2).

Irreversible alteration to the permeability properties was determined from the ratio of the final tritiated water permeability and initial water permeability by calculation of a damage ratio. Following contact with any of the phthalate diesters, a slight increase in the permeability of human skin was detected. In contrast,

relatively large changes in permeability were detected for rat skin, indicating irreversible alteration of the membrane following contact with any of the phthalate diesters tested.

Discussion

In these studies we have measured the *in vitro* absorption of a series of phthalate diesters through human and rat skin (epidermal membranes). This technique is regularly used to determine percutaneous absorption rates, and there is good evidence that the results obtained *in vitro* are predictive of *in vivo* absorption (10). When *in vivo* and *in vitro* measurements are made under identical application conditions and with skin from the same body site, very similar patterns and rates of absorption have been detected (11,12). The *in vitro* absorption rates were measured during a prolonged period of chemical contact with the skin and should be regarded as the maximum rate of absorption through intact skin. Often, absorption data alone are not very meaningful, as very often the rates are influenced by the concentration applied and the vehicle. This criticism is not so relevant, however, to this study, where the neat chemicals have been applied to the skin. Also, the effect of excessive tissue hydration, which in *in vitro* percutaneous absorption experiments is known to influence permeability (13), has been reduced by the design of the cell (donor surface open to atmosphere) and by allowing the epidermal membranes to hydrate naturally before application of the test compound. By using this technique, the permeability of different skin types (body areas or other species) can be measured under precisely the same experimental conditions, thus allowing the quantitation of any differences. The choice of test compound is not restricted, and toxic or radiolabeled molecules may be safely applied to human skin.

The absorption rates of the esters through human and rat skin have been compared with their published or experimentally determined physicochemical properties. The esters showed a wide range of aqueous solubility and lipophilicity. However, they had a narrower range of molecular weights and volume.

Chemical diffusion theory predicts only a slight difference in molecular diffusivity between these mole-

Table 1. Absorption rate data of the *o*-phthalate diesters through human and rat skin.

| Chemical | Permeability constant, ^a × 10 ⁻⁶ cm/hr | | Steady state absorption rate, ^a μg/cm ² /hr | | Lag time, hr | | Damage ratio | |
|----------|---|------------------------|--|------------------------|--------------|-----|--------------|------|
| | Human | Rat | Human | Rat | Human | Rat | Human | Rat |
| DMP | 3.32 ± 0.54 n = 4 | 34.50 ± 3.51 n = 10 | 3.95 ± 0.64 n = 14 | 41.6 ± 4.18 n = 10 | 0.1 | 0.5 | 1.5 | 4.3 |
| DEP | 1.14 ± 0.10 n = 11 | 37.00 ± 8.3 n = 11 | 1.27 ± 0.11 n = 11 | 41.37 ± 9.28 n = 11 | 6.0 | 1.1 | 1.5 | 12.4 |
| DBP | 0.23 ± 0.06 n = 15 | 8.95 ± 0.09 n = 8 | 0.07 ± 0.02 n = 15 | 9.33 ± 0.09 n = 9 | 2.9 | 0.4 | 1.8 | 4.0 |
| DEHP | 0.57 ± 0.12 n = 9 | 2.28 ± 0.23 n = 8 | 1.06 ± 0.23 n = 9 | 2.24 ± 0.23 n = 9 | 3.1 | 3.9 | 2.3 | 9.5 |

^a Permeability constant and steady-state absorption rates are mean values ± SE; n = number of determinations.

Table 2. Physicochemical characteristics of the *o*-phthalate diesters.

| Chemical | Formula | Molecular wt | Molecular vol, Å ³ | Solubility in water, mL/mL × 10 ³ | log ₁₀ P value | |
|------------------------------------|--|--------------|-------------------------------|--|---------------------------|----------|
| | | | | | Tables | Measured |
| Dimethylphthalate (DMP) | C ₆ H ₄ (COOCH ₃) ₂ | 191 | 164 | 3.38 | 1.38 | 1.34 |
| Diethylphthalate (DEP) | C ₆ H ₄ (COOC ₂ H ₅) ₂ | 222 | 200 | 0.78 | 2.47 | 2.47 |
| Di- <i>n</i> -butylphthalate (DBP) | C ₆ H ₄ (COOC ₄ H ₉) ₂ | 278 | 231 | 0.14 | 4.57 | >6 |
| Di-(2-ethyl-hexyl)phthalate (DEHP) | C ₆ H ₄ [COOCH ₂ CH(C ₂ H ₅)C ₄ H ₉] ₂ | 391 | 313 | 0.01 ^a | 8.25 ^b | >6 |

^a Value from (16).

^b Calculated value.

cules. This is based on the relationship between molecular volume (v) range (covering less than a factor of two) and the diffusion coefficient (D_0):

$$D_0 = \frac{\text{Constant}}{v^{-0.5}} \quad (8)$$

The marked changes in the percutaneous absorption of these molecules should be attributed to other physicochemical properties.

The absorption rates of the esters varied both inter- and intraspecies. Under similar experimental conditions, the glycol ethers showed a similar variation (9). This high degree of variability probably reflects normal, intersubject differences as similar ranges are seen in *in vivo* experiments (14). The human skin was less permeable to the diesters than the rat skin, though no constant factor of difference was measured; i.e., DEP (human, less than 30 times rat), and the closest agreement was measured with DEHP (human skin, less than 4 times rat).

A relationship was apparent between the measured rates of absorption and the lipophilicity of the esters (Fig. 1), the rate of absorption through human skin

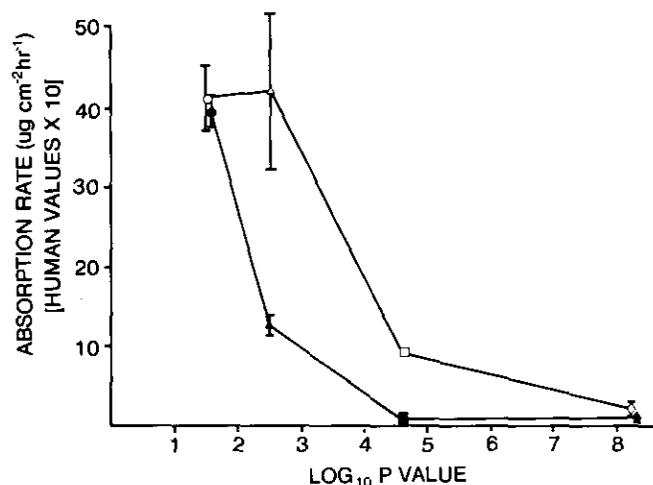


FIGURE 1. Absorption rates of the *o*-phthalate diesters through human and rat skin (human values × 10) plotted against lipophilicity (log₁₀P values). (●) human, DMP; (▲) human, DEP; (■) human, DBP; (◆) human, DEHP; (△) rat, DMP; (○) rat, DEP; (□) rat, DEP; (◇) rat, DEHP.

being decreased with increasing lipophilicity. A similar relationship was detected through rat skin (Fig. 1). The major difference in the absorption trend with lipophilicity between the species was the maintained (relative) high absorption rate of DEP through the rat skin.

A reverse relationship was observed when the absorption rate was plotted against aqueous solubility (Fig. 2). There was a marked increase in absorption with increasing aqueous solubility. DEP absorption through rat skin was comparable to DMP and did not show the decrease obtained with human skin.

This relationship has previously been reported for the percutaneous absorption of neat alcohols through human skin (15). It is proposed that this is due to a decrease in the solubility of the more lipophilic molecules in the stratum corneum limiting the amount of chemical entering the diffusion barrier. It should not be confused with the artificial barrier presented by the dermis to more lipophilic molecules when whole skin is used in *in vitro* experiments. In these reported experiments, epidermal membranes were used, not whole skin. The deviation in the absorption of these phthalate esters

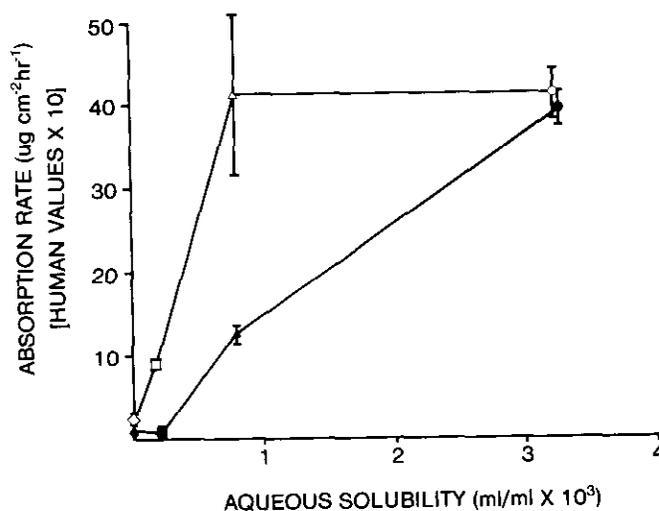


FIGURE 2. Absorption rates of the *o*-phthalate diesters through human and rat skin (human values × 10) plotted against aqueous solubility. (●) human, DMP; (▲) human, DEP; (■) human, DBP; (◆) human, DEHP; (○) rat, DMP; (△) rat, DEP; (□) rat, DEHP; (◇) rat, DEHP.

between the human and rat membranes is to be expected, given the complex differences in the biochemical and structural composition of the two membranes. Interestingly, these chemicals have relatively low systemic toxicity by dermal exposure. There is, however, an apparent relationship between aqueous solubility and the acute systemic toxicity, with the more water soluble esters being more toxic (16). The different percutaneous absorption rates would suggest differences in bioavailability and subsequent differences in toxicity following dermal exposure.

Irreversible alteration in skin barrier function due to contact with the esters was assessed and damage ratios calculated (Table 1). Under similar experimental conditions contact with water alone produced a damage ratio of up to 2 (17). We have previously used this method to assess the effect of a large number of other solvents (9). With human skin, none of the diesters gave damage ratios which could be considered significantly different from that caused by hydration (water). Relative to other organic solvents (9), these phthalate esters caused little irreversible alteration to human epidermal membranes. Following contact of the diesters with the rat skin, alterations in barrier function were noted. This type of alteration might enhance absorption of the chemical, and this would also occur *in vivo*. The increased alteration of rat skin relative to human skin occurred despite a shorter contact period with the phthalate diester under test. We did not study the time course of the induction of this damage and cannot, therefore, compare the changes which occurred in rat and human skin at the same time point. Any enhancement of absorption as a result of damage would be greater for rat skin.

In comparison to other chemicals (5,9), when applied as the neat chemical, the phthalate esters were absorbed slowly through human skin, with rates measured through rat skin higher than through human.

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