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Kinetic Study of Cutaneous and Subcutaneous Distribution Following Topical Application of [7,8-^{14}C]rac-α-Lipoic Acid onto Hairless Mice

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ABSTRACT. To diminish oxidative injury, topically applied antioxidants must reach susceptible cells. α-Lipoic acid is a potent thiol antioxidant that might be useful for skin protection; therefore, its skin penetration kinetics were assessed. The cutaneous and subcutaneous distributions of [7,8-^{14}C]rac-α-lipoic acid were studied in anesthetized hairless mice after application of a 5% solution in propylene glycol for 0.5 to 4 hr. The mice were killed; then the skin was washed, and the stratum corneum was removed by 10 cellophane tape strippings. A punch biopsy of the frozen, stripped skin was sectioned, and amounts of [^{14}C]-α-lipoic acid were determined in strippings and slices of epidermis (4 × 5 μm), dermis, and subcutaneous fat (10 × 10 μm, 20 × 20 μm). The rate of [^{14}C]-α-lipoic acid absorption into skin was constant by 30 min (0.10 ± 0.01 nmol/cm^2/min); maximum skin concentrations were reached by 2 hr. The [^{14}C]-α-lipoic acid penetration kinetics into the first layer of the stratum corneum predicted its penetration through the stratum corneum and subsequent percutaneous absorption (r^2 = 0.96, P < 0.02). Cutaneous absorption of unlabeled α-lipoic acid and its reduction to the more potent antioxidant form, dihydrolipoic acid, were also demonstrated, using HPLC analysis with electrochemical detection. In conclusion, α-lipoic acid topically applied to skin penetrated readily, and was reduced to dihydrolipoic acid. Thus, α-lipoic acid could potentiate skin antioxidant protection. BIOCHEM PHARMACOL 52;4:627–633, 1996.

KEY WORDS. antioxidant; reactive oxygen species; 6,8-dithiooctanoic acid; percutaneous absorption

Reactive oxygen species (ROS) have been implicated in the etiology of skin cancer [1, 2] and photoaging [3, 4]. ROS can oxidize lipids, proteins, or DNA, leading to the formation of oxidized products such as lipid hydroperoxides, protein carbonyls, or 8-hydroxyguanosine [5–8]. Cells are protected against oxidative damage by a variety of enzymatic and non-enzymatic antioxidants [9, 10]. However, if the presence of ROS overwhelms the antioxidant complement of the skin, cell constituents can be damaged severely. This readily occurs during ultraviolet light exposure [11–13].

The exogenous administration of antioxidants is one possible approach to bolster the endogenous antioxidant defense and prevent oxidative injury [14–18]. Since systemic delivery of antioxidants to the target site is inefficient [19, 20], topical application could be beneficial if sufficient quantities of the substance penetrate the skin.

An ideal antioxidant for topical application should (1) have high antioxidant activity, (2) penetrate the skin, (3) be present in its active form in the skin, and (4) protect against oxidative damage in the skin. α-Lipoate and dihydrolipoate (see Fig. 1), a thiol redox couple, are potent antioxidants both *in vitro* and *in vivo* (reviewed in Ref. 21). In vitamin E-deficient animals, α-lipoic acid substitutes for, and interacts with, other antioxidants [22]. Fuchs and Milbradt [23] have demonstrated that intracutaneously injected dihydrolipoate and orally administered α-lipoic acid are effective in protecting skin against inflammation. Topical application of α-lipoic acid, however, is a more practical route of administration.

Penetration of dihydrolipoic acid in murine skin has been evaluated indirectly by measuring changes with spin-labeled dihydrolipoic acid in membrane polarity, using spatial electron spin resonance [24]. However, the physicochemical properties of dihydrolipoic acid may be different from those of spin-labeled dihydrolipoic acid, which contains two covalently bound nitroxides. Furthermore, dihydrolipoic acid is unstable, so it is not useful for routine application to skin.

To evaluate whether α-lipoic acid meets the criteria of an ideal skin antioxidant, we determined the absorption kinetics of [^{14}C]-α-lipoic acid into the epidermis and dermis

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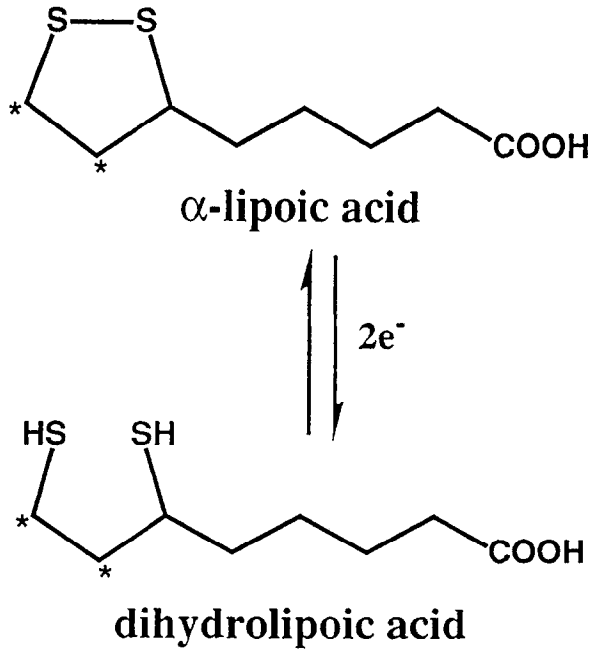

FIG. 1. The redox couple, α-lipoic acid and dihydrolipoic acid.

in vivo, using the method of Schaefer and Stüttgen [25]. In addition, we assessed the penetration of unlabeled α-lipoic acid into skin and its conversion to dihydrolipoic acid by HPLC with electrochemical detection.

MATERIALS AND METHODS

Animals

Mice (female hairless mice, 8- to 10-weeks-old) were purchased from Charles River Laboratories (Wilmington, MA) and kept under standard conditions of temperature and light. Food (Harlan Teklad Rodent Diet, Madison, WI) and water were provided *ad lib.*

[7,8-^{14}C]rac-α-Lipoic Acid Solution

Unlabeled rac-α-lipoic acid and [7,8-^{14}C]rac-α-lipoic acid were provided by ASTA Medica (Frankfurt, Germany). [^{14}C]-α-Lipoic acid was present as the free acid in 0.05 M Tris buffer with a radiochemical purity of 95%, a specific radioactivity of 1283 MBq/mmol (34.7 mCi/mmol), and a concentration of 30.9 mM. The solution for topical application was prepared by adding 2.0 to 2.4 μCi of [^{14}C]-α-lipoic acid to a 240 mM (5%, w/v) solution of α-lipoic acid in propylene glycol (Sigma, St. Louis, MO).

Application Conditions

The mice were anesthetized with two consecutive intraperitoneal injections of sodium pentobarbital (50 mg/kg body weight, Nembutal, Abbott Laboratories, North Chicago, IL). Then a polyethylene ring was glued with silicon (Aquarium Sealant, Dow Corning Corp., Midland, MI) onto the lower back of each animal. [^{14}C]-α-Lipoic acid solution (25 μL) was applied uniformly within the 2-cm^2 ring. Mice remained anesthetized during the entire experimental period of up to 4 hr. After 0.5 hr (N = 5), 1 hr (N = 4), 2 hr (N = 5), or 4 hr (N = 5), the excess substance on the treated area was washed as described by Dupuis *et al.* [26]. Briefly, the skin was rinsed three times with 300 μL of ethanol:water (95:5) followed by two rinses with 300 μL of distilled water and carefully dried with cotton-tipped swabs. Radioactivity in the rinses and the cotton tip was counted.

In a second experiment, 25 μL of a 5% solution of unlabeled α-lipoic acid in propylene glycol (240 mM) was applied to the skin of four mice as described above, and penetration was allowed to take place for 2 hr. These mice were used to determine the amount of α-lipoic acid and dihydrolipoic acid present in whole skin using HPLC (see below).

Histology

In a preliminary experiment, cryosections were obtained from four untreated mice for the purpose of identifying the various layers of skin. Dorsal skin was excised *in toto*; a 6-mm punch biopsy was taken, frozen, and sliced in parallel to the skin surface with a cryomicrotome (Kryostat 1720, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) as described by Schaefer and Stüttgen [25]. The cryosections of skin were hematoxylin/eosin stained and examined by microscopy to identify cell morphology of individual sections. On the basis of this examination, the epidermis was localized to the first four slices of 5 μm thickness. The subsequent ten sections (10 μm) corresponded to papillary dermis.

Paraffin-embedded, hematoxylin/eosin-stained skin was sectioned perpendicular to the skin surface. These sections were examined using an ocular micrometer. Epidermal thickness equaled 20 ± 5 μm (N = 5), similar to previously reported values of epidermal thickness (29–43 μm) in hairless mice [27–30].

Measurement of Cutaneous and Subcutaneous Distribution of [7,8-^{14}C]rac-α-Lipoic Acid

The animals were killed by cervical dislocation. Immediately after washing the skin, the stratum corneum was removed from the skin using cellophane tape (Scotch 810, 3M, St. Paul, MN) ten times to obtain single layers of stratum corneum; hereafter called stripping. Each stripping was placed in a separate vial containing 5 mL of tissue solubilizer (Soluene 350, Packard, Meriden, CT).

The dorsal skin was removed, frozen, and sectioned as described above: epidermis (four slices of 5 μm), papillary dermis (ten sections of 10 μm), and the remaining tissue cut in 20-μm slices. Each slice was placed in a separate scintillation vial containing 3 mL of tissue solubilizer.

Two more punch biopsies were taken to determine the lateral diffusion of the compound. One sample was taken as

close as possible to the application site at the external face of the polyethylene ring and a second biopsy was taken 1.2 cm from the ring. These biopsies were placed in 5 mL of tissue solubilizer.

Tape strippings, tissue slices, and whole skin biopsies were incubated in tissue solubilizer for 24 hr at 40°. After complete digestion, scintillation fluid (Hionic Fluor, Packard) was added, and the ^{14}C was measured by scintillation counting (Beckman LS 5000 TD, Palo Alto, CA). The disintegrations per minute were converted to nanomoles of α-lipoic acid per slice based on the specific activity of the applied material.

Extraction and Detection of Unlabeled α-Lipoic Acid

The extraction and detection of α-lipoic acid in skin were performed as previously described [22]. Briefly, an aliquot of skin (50–100 mg) was ground under liquid nitrogen and subsequently homogenized for 1 min with 1.5 mL of ice-cold 3.3% 5-sulfosalicylic acid and 5 mM EDTA; then 1.5 mL of ethanol was added, and the sample was mixed vigorously for 1 min and centrifuged at 3000 *g* for 3 min. The supernatant either was injected directly into the HPLC or was stored under liquid nitrogen for a maximum of 1 week. In unpublished experiments, we have observed no change in the α-lipoic acid and dihydrolipoic acid contents of stored compared with immediately injected samples.

The α-lipoic acid/dihydrolipoate couple was analyzed by HPLC with a C18 column (10 cm, 3 μm, Microsorb, Rainin Instruments, Woburn, MA) and a mobile phase of water:methanol:acetonitrile (50:30:20) with 5 g/L monochloroacetic acid, and was detected electrochemically with a dual gold/mercury electrode, as described [31, 32]. The upstream generator electrode was set at a potential of −0.900 V, and the downstream detector electrode at +0.050 V. Typical chromatograms, recoveries, and the HPLC set-up have been described [31, 32]. During extraction and directly before injection, special care was taken to remove oxygen, which interferes with the reduction, by constantly sparging the solvents and buffers with helium.

Standards of α-lipoic acid and dihydrolipoic acid were prepared by dissolving known weights of authentic compounds in monochloroacetic acid. Concentrations of α-lipoic acid were verified by spectrophotometry at 330 nm. Standards were stored at −200°. The instrument has a roughly 4-fold greater sensitivity for dihydrolipoate than for lipoic acid. A concentration range of standards that encompassed that of the samples was used routinely. Standards were injected at 2-hr intervals during sample measurements to correct for electrode deterioration and baseline drift.

Statistics

Statistical significance was determined by two-factor ANOVA and individual comparisons using the least squares means test. A $P < 0.05$ was considered significant. All data in text and figures are expressed as means ± SD.

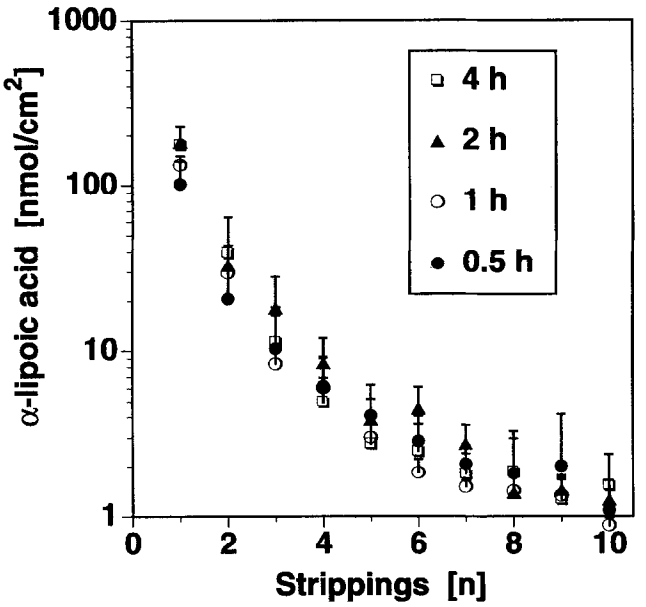

FIG. 2. Distribution of [^{14}C]-α-lipoic acid in ten tape strippings of stratum corneum at 0.5, 1, 2, and 4 hr following topical application of 25 μL of 5% [^{14}C]-α-lipoic acid in propylene glycol to skin of hairless mice. Shown are the means ± SD; N = 5 mice at all time points, except for 1 hr where N = 4.

RESULTS

Penetration of [^{14}C]-α-Lipoic Acid into the Stratum Corneum

[^{14}C]-α-Lipoic acid, measured in ten tape strippings of skin from mice following topical application, decreased exponentially from stripping 1 to 10 (Fig. 2). At each of the time points, strippings 1 and 2, which represent the first layers of stratum corneum, contained significantly higher ($P < 0.0001$) [^{14}C]-α-lipoic acid concentrations than the other strippings. Notably, in each of the strippings at the different times (0.5, 1, 2, and 4 hr) [^{14}C]-α-lipoic acid concentra-

TABLE 1. [^{14}C]-Lipoic acid concentrations in strippings of stratum corneum

	[^{14}C]-α-Lipoic acid concentrations (nmol/cm²)			
	Application time (hr)			
Strip	0.5	1.0	2.0	4.0
1	101 ± 17	133 ± 8	182 ± 20	176 ± 23
2	21 ± 4	30 ± 3	33 ± 5	39 ± 11
3	10 ± 3	8.4 ± 0.9	18 ± 4	11 ± 3
4	6.2 ± 1.3	6.0 ± 1.4	8.5 ± 1.5	4.9 ± 0.9
5	4.1 ± 1.0	3.0 ± 0.4	3.9 ± 0.6	2.8 ± 0.5
6	2.9 ± 0.5	1.8 ± 0.2	4.5 ± 0.7	2.5 ± 0.5
7	2.1 ± 0.3	1.5 ± 0.1	2.8 ± 0.4	1.8 ± 0.3
8	1.8 ± 0.7	1.4 ± 0.2	1.4 ± 0.2	1.9 ± 0.5
9	2.0 ± 1.0	1.3 ± 0.1	1.4 ± 0.1	1.3 ± 0.2
10	1.1 ± 0.2	0.9 ± 0.1	1.3 ± 0.1	1.5 ± 0.4
Sum	152 ± 52	188 ± 19	257 ± 62	243 ± 29

Values are means ± SD; N = 5 mice per time point, except for 1 hr where N = 4.

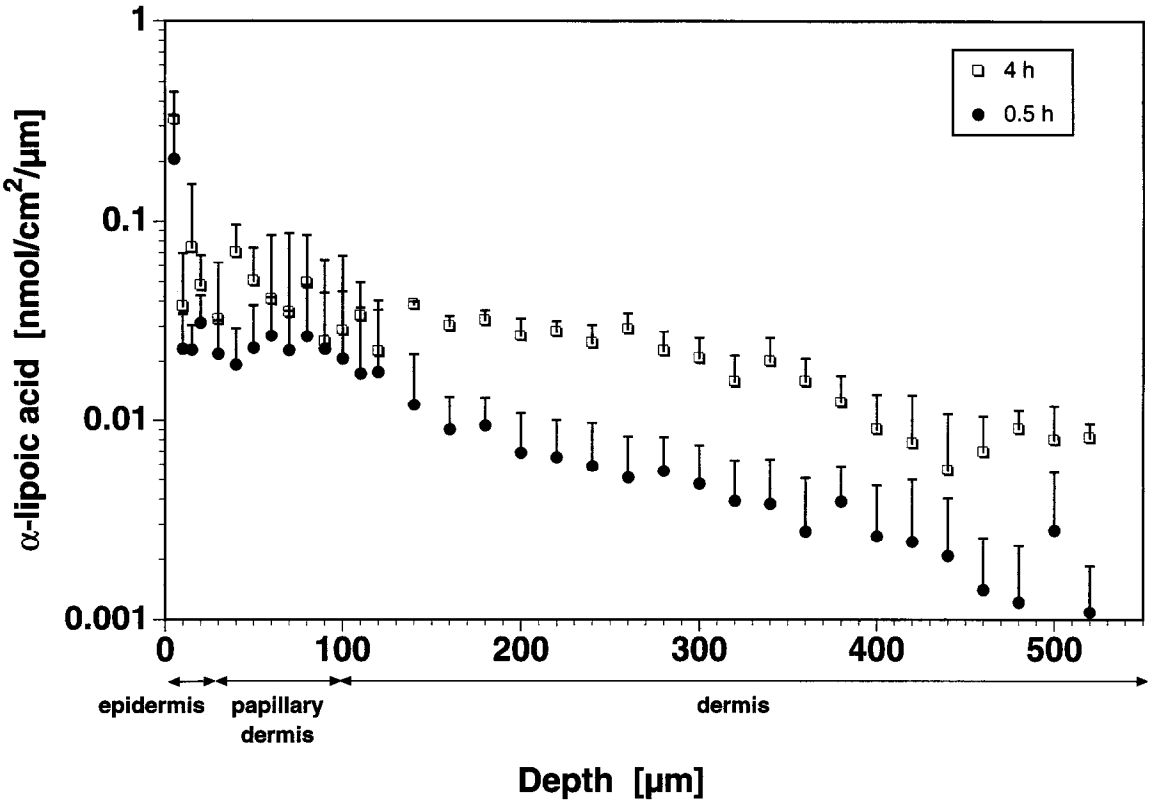

FIG. 3. Concentrations of [^{14}C]-α-lipoic acid in the skin beneath the stratum corneum of hairless mice at 0.5 and 4 hr following topical application, as described in Fig. 2. To normalize for different slice thicknesses all data are expressed in nmol/cm^2/μm. Shown are the means ± SD, N = 5.

tions were not statistically different. Shown in Table 1, total [^{14}C]-α-lipoic acid in the strippings increased markedly for up to 2 hr; concentrations were similar at 2 and 4 hr.

Penetration of [^{14}C]-α-Lipoic Acid into Skin beneath the Stratum Corneum

To evaluate the penetration of [^{14}C]-α-lipoic acid into the skin below the stratum corneum, the [^{14}C]-α-lipoic acid was measured in 5–20 μm slices of the skin biopsies at four time points (0.5, 1, 2, and 4 hr). Five mice were used per time point (0.5, 2, and 4), except for 1 hr, where N = 4; the number of cuts per mouse varied from 28 to 40; at 0.5 hr the numbers of cuts were 32 ± 4, at 1 hr 34 ± 5, at 2 hr 33 ± 3, and at 4 hr 31 ± 3. Figure 3 shows data from the 0.5- and 4-hr time points; data at 1 and 2 hr were intermediate—these gradients are not shown. At all time points, [^{14}C]-α-lipoic acid was detectable in the epidermis, dermis, and subcutaneous fat to a depth of 500 μm. The first slice contained the highest [^{14}C]-α-lipoic acid concentrations; the successive cuts from the next 100 μm of the skin biopsy contained relatively constant concentrations—at 0.5 hr 0.024 ± 0.003 nmol/cm^2/μm, at 1 hr 0.046 ± 0.01, at 2 hr 0.069 ± 0.036, and at 4 hr 0.045 ± 0.016. The deeper portions of the skin contained exponentially lower [^{14}C]-α-lipoic acid concentrations.

To estimate overall substratum corneum [^{14}C]-α-lipoic acid penetration, its concentrations in the skin fractions were summed. These were 5.4 ± 3.1 nmol/cm^2/μm at 30 min, 9.6 ± 2.3 at 1 hr, 14.8 ± 4.8 at 2 hr, and 13.3 ± 5.3 at 4 hr. These data demonstrate that maximum skin α-lipoic acid concentrations were achieved by 2 hr.

Comparison of the Penetration of [^{14}C]-α-Lipoic Acid into the Stratum Corneum and the Underlying Skin

[^{14}C]-α-Lipoic acid concentrations in stratum corneum, in residual epidermis, and in the dermis and subcutis are compared in Fig. 4. An apparent maximum was reached at 2 hr in each of these compartments. At 2 and 4 hr after topical application, of the [^{14}C]-α-lipoic acid recovered in the skin ~95% was located in the stratum corneum, ~1% in the residual epidermis, and ~4% in the dermis and subcutaneous fat (Fig. 4).

The stratum corneum [^{14}C]-α-lipoic acid concentrations were predictive of those in the underlying skin. A linear correlation (r^2 = 0.98) was observed between [^{14}C]-α-lipoic acid in tape strippings and in residual skin (Fig. 5a). Although [^{14}C]-α-lipoic acid concentrations in the first two tape strippings also correlated with that in the residual skin (Fig. 5b), the [^{14}C]-α-lipoic acid concentrations in tape strippings 3–10 were not similarly correlated (Fig. 5c).

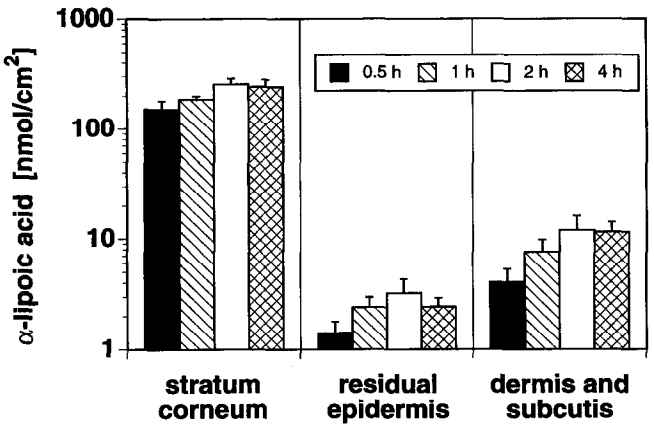

FIG. 4. Compartmental localization of [^{14}C]-α-lipoic acid in the skin of hairless mice at 0.5, 1, 2, and 4 hr following its topical application, as described in Fig. 2. Shown are the means ± SD; N = 5 mice at all time points, except for 1 hr where N = 4.

Lateral Diffusion of α-Lipoic Acid

To evaluate the lateral diffusion of [^{14}C]-α-lipoic acid, biopsies were taken directly adjacent to the application site, as well as 1.2 cm further away. The ^{14}C-contents of the adjacent biopsies were not above background levels and were not statistically different from those of a more distant biopsy (data not shown). Thus, lateral diffusion of [^{14}C]-α-lipoic acid was insignificant as compared with its penetration into the application site.

Reduction of α-Lipoic Acid to Dihydrolipoic Acid

To evaluate the redox state of α-lipoic acid following skin penetration, α-lipoic acid and dihydrolipoate concentrations were measured using HPLC with electrochemical detection. At 2 hr following topical application, total (oxidized and reduced) α-lipoic acid concentrations in mouse skin were 300 ± 122 nmol/g skin (N = 4). About 5% of α-lipoic acid (15 ± 8 nmol/g skin) was present in the reduced form, dihydrolipoate. Total α-lipoic acid concentrations measured by HPLC were similar to those estimated from the applied [^{14}C]-α-lipoic acid (350 ± 48 nmol/g skin, based on the assumption that the weight of hairless mouse skin is 39 ± 4 g/cm^3).

DISCUSSION

This study demonstrates that α-lipoic acid readily penetrated murine skin *in vivo*. By 30 min, an appreciable amount of [^{14}C]-α-lipoic acid was detected not only within the stratum corneum, but also in the dermis and subcutaneous layers. Similar distribution profiles for other molecules have been reported previously [25, 33, 34].

Our data support the concept that a constant flux of [^{14}C]-α-lipoic acid through the skin was established rapidly. As early as 30 min, the first stripping of stratum corneum contained 100 times more [^{14}C]-α-lipoic acid than the last stripping; intermediate strippings contained exponentially decreasing amounts. Tsai *et al.* [35] observed for application times of 1–8 hr similar distribution profiles of minoxidil into the stratum corneum of hairless mice. They suggested that the concentration gradients between layers were established within the first hour. Rougier *et al.* [36] observed *in vivo* that the lag time (the intercept of the straight line representing the constant flux with time) for the appearance of the concentration gradients was short (11 ± 2 min) and very similar for substances with very different physicochemical properties, such as benzoic acid and insulin.

The first epidermal slice contained higher concentrations of [^{14}C]-α-lipoic acid than did the other slices (Fig. 3). This could be due either to an actual higher concentration in the upper epidermis as described for vitamin A by Schaefer *et al.* [37] or contamination of the sample with stratum corneum. Although no stratum corneum was vis-

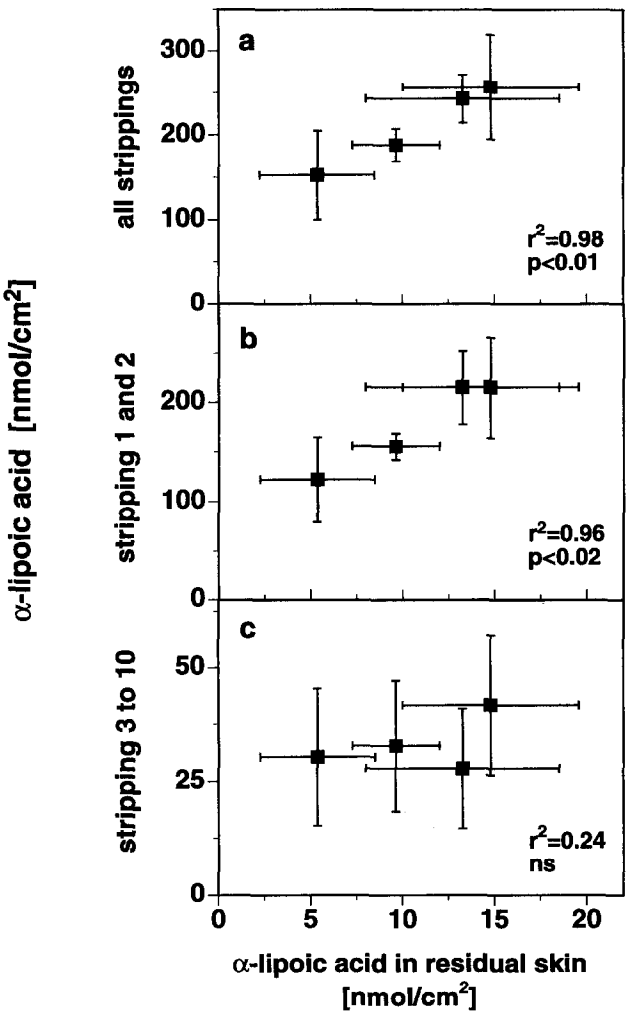

FIG. 5. Correlations between the concentrations of [^{14}C]-α-lipoic acid in the stratum corneum and those in the residual skin. Shown are the means ± SD; N = 5 mice at all time points, except for 1 hr where N = 4. (a) Entire stratum corneum (tape strippings 1–10) vs residual skin; (b) first and second tape strippings vs entire skin; and (c) 3–10 tape strippings vs entire skin.

ible in histologic analysis, we cannot rule out completely the latter possibility.

The cuts from the papillary dermis contained nearly uniform concentrations of [^{14}C]-α-lipoic acid (Fig. 3), similar to studies of linoleic acid [38] or benzoyl peroxide [39]. α-Lipoic acid might partition into the papillary dermis because sebaceous glands located in this region are lipid-rich and α-lipoic acid is only slightly hydrophilic (partition coefficient P_{ow} = 0.34 (log P_{ow} = −0.47) in *n*-octanol/water at pH 7.4 [40]). The affinity of [^{14}C]-α-lipoic acid for lipophilic regions can also explain its high concentration in the stratum corneum, relative to the epidermis, dermis, and subcutaneous tissue.

The pattern of [^{14}C]-α-lipoic acid appearance into stratum corneum resembles its pattern of appearance in the various skin compartments (Fig. 4). This relationship was explored in panels a and b of Fig. 5, where the [^{14}C]-α-lipoic acid concentrations in the first and second tape strippings, or in the entire stratum corneum, were correlated with [^{14}C]-α-lipoic acid concentrations in the skin. Fick's first law can be applied to the stratum corneum, since the distribution of lipoate is unchanged at all time points (Fig. 2); an infinite dose condition is present on the skin surface and a sink in the lower stratum corneum. If Fick's first law is applied to the stratum corneum, the flux of absorption is essentially dependent on the amount of [^{14}C]-α-lipoic acid present in the uppermost layers of the stratum corneum. We propose from our experimental data that in addition to being able to use tape strippings of the stratum corneum to predict the percutaneous absorption of a substance [41], the kinetics of cutaneous absorption can be also predicted from the contents of tape strippings of the stratum corneum, the first ones being the most relevant.

The measurements of α-lipoic acid by HPLC demonstrate that once α-lipoic acid has penetrated into the skin it is reduced to its more potent antioxidant form, dihydrolipoic acid. Although the concentrations of lipoic acid estimated from the radioactivity or from HPLC analyses were similar, the location of the reduction of α-lipoic acid to dihydrolipoic acid cannot be determined from this study.

This study presents the penetration kinetics of α-lipoic acid into murine skin and demonstrates that α-lipoic acid enters the skin rapidly. Thus, α-lipoic acid is a good candidate for topical application to protect against oxidative stress. It should be emphasized that our experiment was carried out in hairless mice, and caution should be taken in extrapolating the findings to human skin [42, 43].

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