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INVESTIGATIVE REPORT

In Vitro Permeation of Nickel Salts Through Human Stratum Corneum

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Allergic contact dermatitis due to nickel salts is common. It is therefore important to measure the permeation of these salts through the stratum corneum (SC), the primary rate-limiting domain in skin. An advanced diffusion system and analytical techniques now enable better measurement of the flux than was possible in earlier experiments. Human SC was prepared by trypsinization of dermatomed cadaver leg skin. The diffusion system included diffusion cells with a spiral line. Aqueous solutions of nickel salts ($Ni(NO_3)_2$, $NiSO_4$, $NiCl_2$ and $Ni(^-OOCCH_3)_2$ at 1% Ni^{2+} concentration) were used as the donor solution (400 μL/cell). The receptor fluid, pure water, was collected up to 96 h after application of the donor solutions. Nickel concentrations in the donor and receptor fluid, as well as in the SC, were analysed using inductively coupled plasma mass spectrometry (ICP-MS) with a confidence limit of 0.5 ppb. Based on the total recovery of nickel from the experiments, about 98% of the dose remained in the donor solution, whereas 1% or less was retained in SC and less than 1% was found in the receptor fluid. Following an early surge, nickel permeates slowly across SC. The steady-state permeability coefficients of nickel were calculated from the flux data (approximately $5.2–8.5 \times 10^{-7}$ cm/h) with no significant difference among the salts. The results concur in principle with earlier studies conducted using the full-thickness human skin *in vitro*, and suggest that *in vivo* nickel ions may permeate simultaneously by routes of diffusion such as the shunt pathway, apart from slow transcellular/intercellular diffusion alone. ***Key words: human; in vitro; nickel salts; percutaneous absorption; stratum corneum.***

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INTRODUCTION

In the fields of environmental and occupational health and safety, percutaneous absorption is particularly important when assessing the risk of human exposure to toxics. *In vitro* percutaneous studies can be used to define the diffusion characteristics of xenobiotics as one way of documenting their bioavailability. For several heavy metals, and nickel in particular, gathering data on skin penetration in a quantitative manner seems to meet with serious technical obstacles. On reviewing the literature, it is evident that researchers have followed the most disparate procedures when conducting those experiments, thus making comparisons across studies difficult (1).

As a well-known irritant and allergen, nickel causes both immediate and delayed, specific and non-specific type allergy reactions following dermal exposure (2–4). The dermatotoxicology of nickel compounds presents a major scientific challenge owing to the apparent paradoxes observed in its behavior in contact with the epidermis, so several significant issues await resolution reconciled through appropriate experimentation. To date, the most comprehensive study of skin permeation by inorganic nickel salts was performed *in vitro* on full-thickness skin (5). The present study focuses only on the permeation of nickel from various salts across the uppermost layer of the epidermis, the stratum corneum (SC). With the aid of sophisticated analytical methods such as inductively coupled plasma mass spectroscopy (ICP-MS) (6, 7), it is now possible to accurately detect ultra-trace amounts of the metal in biological substrates, such as SC tissue or the donor and receptor phases from *in vitro* studies, yielding a sufficiently accurate picture of metal adsorption and absorption.

This investigation was designed to clarify the effect the counter ions sulfate, chloride, nitrate and acetate may have on *in vitro* diffusion of aqueous nickel solutions applied at a 1% nickel concentration level. We measured respective steady-state flux values under the most conclusive experimental conditions possible. Using human SC separated by trypsinization, the permeation rate of nickel was determined by means of ICP-MS; mass balance was attempted by summation of nickel recovered from the surface, permeant residue present in the SC matrix, and nickel recovered in the receptor phase.

MATERIALS AND METHODS

Preparation of stratum corneum samples

Preparation of stratum corneum samples is a modification of the Tanojo et al. (8) procedure. Human skin obtained from cadaver or surgical operation was spread on an aluminum sheet; the surface was wiped clean using a tissue paper soaked in purified water and the skin was spread, dermal side down, on a Whatman paper soaked in a 0.05% w/w Trypsin Type III (from bovine pancreas, Sigma Chemicals, St. Louis, USA) solution in 0.15 M phosphate buffered saline (PBS, pH 7.4, Sigma). The skin was incubated with the enzyme solution for 2 h at 37°C. SC was then carefully separated from the underlying epidermis using tweezers. Remaining trypsin activity was blocked by submerging and shaking the SC sheet in 0.05% w/w Trypsin Inhibitor Type II (from soybean, Sigma Chemicals, St. Louis, USA) solution in distilled water. Without this inhibition, the remaining trypsin may continue to digest SC and thereby change the SC barrier property. The SC sheet was subsequently washed twice in purified water, dried and stored above silica gel at room temperature.

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Diffusion experiment

Human SC sheet was clamped into a continuous flow-through diffusion cell system maintained at 37°C. The diffusion cells were made of Teflon, with 0.552 cm^2 surface area of exposed skin. The characteristics of this type of cells have been described in detail (9). The donor solutions were aqueous solutions of (a) nickel nitrate [$Ni(NO_3)_2.6\ H_2O$], (b) nickel sulfate [$Ni(SO_4.6\ H_2O$], (c) nickel chloride [$NiCl_2.6\ H_2O$], and (d) nickel acetate [$Ni(CH_3COO)_2.4\ H_2O$]; each contains 1% w/v nickel. The receptor fluid, HPLC-grade pure water, was pumped at a rate of 4–5 ml/h through the receptor side of the diffusion cell and was then collected every 4 h up to 96 h.

Sample preparation and analysis

Donor solutions were recovered from each diffusion cell by flushing 5 times with 1 ml water. These samples, and the receptor fluid samples, were analyzed without pretreatments. The SC sheets from each diffusion cell were boiled in 10 ml 30% hydrogen peroxide solution for 4 h and cooled prior to analysis.

Analysis was performed at the Lawrence Berkeley National Laboratory. All nickel concentrations were determined on VG Elemental Plasma Quad III Inductively Coupled Plasma – Mass Spectrometer (ICP-MS) (7). The ICP-MS reported data are an average of triplicate analysis of mass 60 for nickel. Mass 60 was chosen before mass 62 for reporting because it is more abundant. The plasma and mass spectrometer conditions were optimized using a ^{59}Co solution to optimize the flow rates of the argon gases and the electrical current applied to the different focusing plates of the mass spectrometer. The average number of counts for a 6.0 µg/L ^{59}Co solution was 1.0×10^5 versus 2.0×10^0 to 1.0×10^1 for background at mass 59. The RF power was kept at 1300 Watts. The autosampler parameters were set as follows: read delay of 60 sec, uptake time of 60 sec, and an instrument washout between samples of 200 sec with 3% nitric acid.

The data were collected under a rigorous quality control protocol: sample, blank and midpoint standard were re-analyzed every 10 samples and a secondary check sample was re-analyzed every 20 samples. The sample results were within 5% relative percent difference and the standard and secondary check samples were within 10% of the expected values. The blank was below the detection limit. If any of these QA/QC parameters were not met, the sample group was re-analyzed. The confidence limit for nickel by the ICP-MS method was 0.5 ppb, a factor of 5 above the instrument detection limit of 0.1 ppb. The results are reported as µg/L of nickel.

Data analysis

Sample analysis data were transferred by hand to a spreadsheet (Quattro Pro 7.0 for Windows 95) and the percentage of compound recovered from the applied dose was determined. The permeation profile of nickel from each salt was plotted against time using Microcal Origin and the permeability constant of nickel was calculated from the permeation rate during the steady-state condition. Statistical analyses of the data were performed using the paired Student *t*-test on Microcal Origin.

RESULTS

The results show a relatively minute permeation of nickel salts across human SC (Table I). From the total recovery of nickel after the experiments, less than 1% of the applied nickel dose permeates across the SC, while 1% or less is retained in the SC. Most of the nickel (~98% of the dose) remains in the donor solution on the surface of SC. Retention in the SC is significantly ($p < 0.1$) the highest for the nitrate salt, followed by sulfate and chloride, and lowest for acetate. On the other hand, the amount of nickel from the acetate which permeates the SC does not significantly differ from other salts.

Fig. 1 shows the profile of the permeation rate for all nickel salts: the highest permeation occurred only in the earlier hours up until a certain equilibrium was reached and the flux became steadier. In this early phase, the permeation profiles from each nickel salt clearly differ. While nickel from acetate and chloride salts reached the peak of permeation within 4 h, nickel nitrate peaked after 8 h and nickel sulfate after 12 h post-application. After 24 h, the steady state was obtained and the permeability constants for nickel salts could be calculated from the average permeation rates. The nickel permeation apparently continues for more than 96 h at a low flux.

The permeability constant of nickel salts (Table II) ranges between 5 and 8.5×10^{-7} cm/h, with the highest permeation shown by sulfate, followed by chloride, nitrate and acetate. Statistical analyses, however, show no significant difference among the mean values ($p < 0.05$). These constants are calculated from the values at time range 24 to 96 h.

DISCUSSION

Despite its prominent role as an allergen, quantitative reports on nickel percutaneous absorption *in vivo* and *in vitro* are few and appear contradictory. Nørgaard first demonstrated the absorption of nickel through normal human skin using the "disappearance method". In 24 h, the radiation from protected, dried aqueous deposits of $^{57}NiSO_4$ on the skin at several body sites decreased by about two-thirds. In similar experiments, radiation from $^{57}NiCl_2$ in lanolin decreased by 61% in 41 h (10). However, the loss can be due to the external disposition from skin by mechanical forces, instead of true permeation.

While nickel undeniably does penetrate to the viable epidermis, witness the ease with which it elicits allergic reactions; experimental measurement to quantify actual degree of penetration has given minute and variable values, presumably due to nickel's reactivity and retention in the SC (11–13). Most striking are long induction or lag times seen *in vitro* (5), and the difficulty in finding a significant level of permeant in the receptor phase. In diffusion experiments with $NiSO_4$ through heat-separated human epidermis (14) after 17 h only 1 of 6 cells yielded measurable levels of nickel in the receptor phase, and after 90 h 2 of the 6 cells at no time presented measurable amounts in the receptor chamber. In that experiment, estimated permeability coefficients were 0.03×10^{-4} to

Table I. *The permeation data of nickel salts across human stratum corneum* in vitro *at 96 h*

		% Penetrated	% Donor recovered	% in SC	Total recovery (%)
$NiSO_4.6\ H_2O$	Nickel sulfate	1.09	96.90	0.56	98.55
$NiCl_2.6\ H_2O$	Nickel chloride	0.74	98.73	0.18	99.66
$Ni(NO_3)_2.6\ H_2O$	Nickel nitrate	0.54	82.53	0.95	84.02
$Ni(CH_3COO)_2.4\ H_2O$	Nickel acetate	0.02	105.37	0.10	105.48

Donor concentration: 10 mg/ml Ni^{2+} in each salt. Experiment performed at 37°C.
SC = stratum corneum.

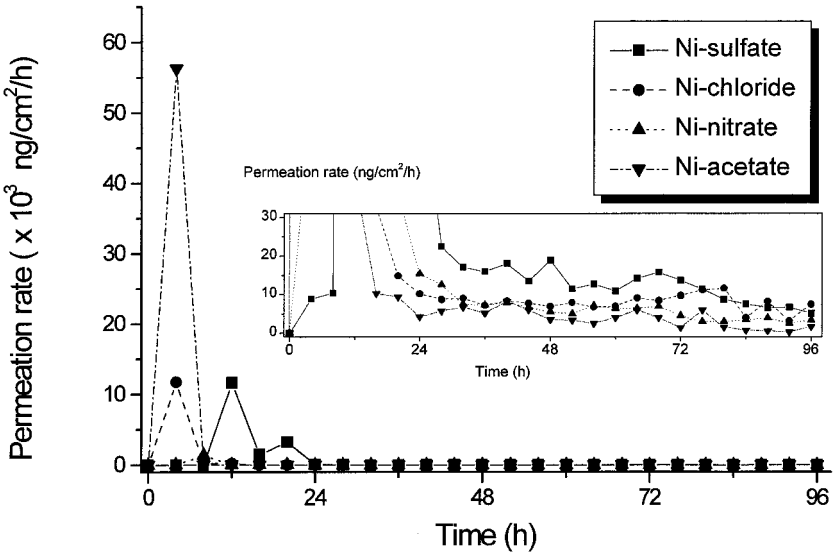

Fig. 1. The 96-h *in vitro* permeation profile of nickel from 4 different salts across human stratum corneum. (■) Nickel sulfate, (●) nickel chloride, (▲) nickel nitrate, (▼) nickel acetate. Inset: the same graphs at a three-magnitude-lower y-axis scale (note the unit).

Table II. *The permeability constants of nickel salts across human stratum corneum* in vitro

		Permeability constant		
		cm/h $\times 10^{-7}$	SD $\times 10^{-7}$	*n*
$NiSO_4.6\ H_2O$	Nickel sulfate	8.5	5.5	3
$NiCl_2.6\ H_2O$	Nickel chloride	6.8	1.2	4
$Ni(NO_3)_2.6\ H_2O$	Nickel nitrate	5.2	1.6	7
$Ni(CH_3COO)_2.4\ H_2O$	Nickel acetate	5.2	1.1	4

Donor concentration: 10 mg/ml Ni^{2+} in each salt. Experiment performed at 37°C.
SD = standard deviation, *n* = number of experiments.
No significant difference between values ($p < 0.05$).

0.1×10^4 cm/h, approximating the counter current effect of SC desquamation.

In contrast to the heat-separated epidermis, the SC in the *in vitro* experiments lacks appendages. The holes/shunts left by the hair follicles and glands shut upon hydration and swelling of the corneocytes (absorption of water into the cells) as can be observed visually by light microscope. This can also be shown by the low rate of permeation of compounds – for instance, nickel – across isolated SC, which would be high if holes or pores exist. The steady-state permeation constant (Kp) calculated in this study may therefore be attributed solely to the diffusion across the transcellular/intercellular barrier.

Using the technique available to date to quantify the low nickel concentration, the present study confirms the low permeation of nickel stated in earlier studies. The highest rate of nickel permeation from the salt solutions occurred primarily in the first 24 h. This was apparently due to a high driving force of concentration gradient when the donor solution was applied for the first time on SC in occlusion. In full-thickness skin, the surge of permeation in early phases can also be observed for compounds with high permeation rate and applied in occlusive systems (17). Since full-thickness skin and heat-separated epidermis consist of various multilayers (or multistrata), the surge cannot always be observed, as the effect can be minimized by the permeation lag time across layers underneath SC. This may explain the discrepancy in lag time with other studies using different skin components. The surge of nickel permeation across SC was short-lived and the amount permeated was low; the total permeation of nickel after 96 h of diffusion was less than 1% of applied dose. Apparently, SC can be quickly saturated with the permeant and reaches an equilibrium where the barrier function limits the permeation to a steady state.

The role of the counter ions on the percutaneous absorption rate with the irritation potential of various nickel salts seen as indicators of penetration is another salient characteristic of nickel, demonstrated in human skin *in vivo* and *in vitro*. Comparison of equimolal nickel salt concentrations showed a clear dose–response relationship and difference in biological effect in volunteers, whereby the chloride and the nitrate were more irritating than the sulfate (3). Also, in the *in vitro* percutaneous studies through full-thickness human skin, nickel chloride permeated at 50 times the rate of nickel sulfate (5). Under all experimental conditions, lag times for sulfate or chloride were substantial (approximately 50 h). (5). After lag times of about 50 h in experiments lasting 144–239 h, occluded $NiCl_2$ entered the receptor fluid about 5–40 times more rapidly than $NiSO_4$, $NiCl_2$ with added Na_2SO_4 or $NiSO_4$ with added NaCl. Without occlusion, the permeation of nickel decreased by more than 90%. It is still not clear whether the barrier quality of full-thickness skin can be maintained for more than 5 days. Nevertheless, the results suggest that the permeation rate of nickel may be influenced by the physical properties of the anions.

In the present study, the permeation constant (across SC) of nickel from chloride salt is not significantly higher than from sulfate, nitrate or acetate salts. However, the permeation

profiles of the first 24 h are different. Nickel chloride and nickel acetate reached the peak of the early surge within 4 h, followed by nickel nitrate after 4 h and nickel sulfate 8 h later. This should contribute to the difference observed in the above-mentioned long-term studies. One may have to look at the effect of water solubility and the ionization properties of the respective salts as they affect ion-pairing in order to understand the real mechanism behind these observations. As shown in solubility data in water and in octanol (Table III), nickel sulfate has low solubility both in water and in n-octanol among the four nickel salts tested. Therefore, in the aqueous donor solution, nickel sulfate is present most in unionized form compared to other salts. In this form, nickel salt bulk molecules might not permeate at a high rate because of the molecular size, albeit without the retention of ion-binding. Being smaller in size, free nickel ions may permeate faster than the unionized molecule. Hence the more nickel ions that exist, the more permeation that may be expected. On the other hand, nickel ion is probably bound to fatty acids, peptides or other counter ions available in the SC. This may increase retention. The net permeation rates are therefore defined by the combination of these effects. Nickel chloride apparently permeates this way, so it reaches the peak of permeation quickly. Nickel nitrate has a high solubility in water and n-octanol and therefore also a higher degree of ionization, which may increase the bonding with SC components, and hence has higher retention in SC. Nickel acetate is an interesting case because its solubility in water is low while it is high in n-octanol. In theory, this should induce more partition into the lipophilic SC than in the donor solution. This could be the reason for nickel acetate reaching the permeation peak in the early phase, but later has the least retention in and total permeation across the SC.

In the deeper layers of epidermis, the water concentration increases and the ionized form of nickel may permeate faster. The combination of the permeation rate in the SC and epidermis (with the assumption of high permeable dermis) will ultimately determine the resulting permeation rate across the full-thickness skin and account for the difference of permeation shown in earlier studies. Apart from that, one reason why analysis of the literature data on skin diffusitivity of nickel remains inconclusive is that different mechanisms appear to operate in its skin penetration and the polarity of the compounds studied, becoming evident depending on the experimental conditions (*in vivo* versus *in vitro*) used. As the metal shows special affinity for keratin with a severe retarding effect on penetration, absorption through the appendages (sweat ducts, follicles and sebaceous glands) must also be invoked as an alternative explaining the pronounced skin reactivity upon contact. Remember that the total area occupied by the appendages is very minute compared to the surface area of the corneocytes (19). While passage through the appendages may contribute to the total permeation, it is not yet clear what part that plays in the onset of reaction.

The donor concentration is standardized at 1% (= 10 mg/ml) nickel level, as this level permits elicitation of allergic contact dermatitis (Uter et al., 1995; Rudzki et al., 1997).

Table III. *Solubility of nickel salts in water and in n-octanol at room temperature*

		Solubility	
		in water[a] (g/100 ml)	in n-octanol[b] (μg/l)
$NiSO_4.6\ H_2O$	Nickel sulfate	62.5	2.07
$NiCl_2.6\ H_2O$	Nickel chloride	254.0	11,366
$Ni(NO_3)_2.6\ H_2O$	Nickel nitrate	238.5	24,283
$Ni(CH_3COO)_2.4\ H_2O$	Nickel acetate	16.7[c]	1822

[a] *CRC Handbook of Chemistry and Physics*, 72nd edn. Boca Raton: CRC Press, 1992.
[b] Hostynek et al., submitted for publication.
[c] *The Merck Index*, 12th edn. Merck & Co. New Jersey: Whitehouse Station, 1996.

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