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EFFECTS OF NITROGEN DIOXIDE ON RESPIRATORY TRACT CLEARANCE IN THE FERRET

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During growth and development, young children are periodically exposed to relatively high concentrations of various air contaminants, including tobacco smoke and environmental pollutants generated by fossil fuel use. The effects of these exposures on respiratory function and lung development are difficult to determine because of interindividual variation and lack of accurate dosimetry. To provide information on the effects of chronic exposure to a common indoor and outdoor pollutant during lung development, a study was performed to assess the effects of exposure to two concentrations of nitrogen dioxide (NO_2; 0.5 or 10 ppm) on tracer particle clearance from the airways of ferrets exposed during postnatal respiratory tract development. Separate groups of ferrets were exposed nose-only to the test atmospheres or clean air 4 h/d, 5 d/wk, for either 8 or 15 wk. Those animals exposed for 8 wk were subsequently housed in a filtered air environment until the particle clearance measurements commenced at 3 wk prior to the end of the 15-wk exposure protocol. Radiolabeled (^{51}Cr) tracer particles were deposited in the respiratory tract of all animals by inhalation, and the clearance rates from the head and thoracic regions were separately monitored for 18 d. No significant effects of the NO_2 exposure on head airways clearance were seen. In contrast, the rates of particle clearance from the thorax of both the 8- and 15-wk groups exposed to 10 ppm NO_2 were significantly reduced, and did not differ from each other. Thoracic clearance was also reduced in animals exposed to 0.5 ppm, but the rate was not significantly different from that of the clean air exposed controls. These results show that NO_2 at moderate concentrations caused highly significant changes in the deep lung of the juvenile ferret, and suggest that impairment of the clearance function may be only slowly recovered after chronic exposure.

Nitrogen dioxide (NO_2) is a toxic gas commonly present in certain occupational settings (e.g., arc welding areas, automobile repair shops) and also at lower concentrations in the ambient environment and in residences where smokers are present and where gas is used for cooking and/or heating (Angle, 1988; Marbury et al., 1988). Many laboratory studies have established the effects of acute and chronic exposures to this gas on lung morphology and function, but few have addressed the question of the effects of repeated exposure during the period of rapid lung growth in neonatal or juvenile animals. These studies are especially important because children are often exposed to NO_2 in their residential or outdoor environment, beginning shortly after birth and continuing until adulthood. For example, in

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homes in which cooking and heating are done with gas or oil stoves, NO_2 concentrations up to about 0.5 ppm have been reported (Angle, 1988) and, with inadequate ventilation conditions, concentrations exceeding 1 ppm could occur (Leaderer, 1982). In the outdoor environment, episodes of high pollution occur in which NO_2 concentrations may approach 0.5 ppm. During 1991 in certain areas of the Los Angeles basin, peak levels of 0.38 ppm were recorded, and concentrations exceeding 0.1 ppm were common during the winter months (California Air Resources Board, 1991). Adverse effects of repeated exposure to NO_2, along with other environmental pollutants, are suggested in the report of Sherwin (1991) showing tissue damage in the small airways of young persons in the Los Angeles area. This lung damage may be expected to compromise the effectiveness of crucial respiratory tract defense systems, increasing the potential for both acute and chronic infectious disease. Children may be especially vulnerable to the harmful effects of this pollution because the lung is still developing, and they are usually physically active, resulting in increased lung ventilation and delivered dose of the pollutants.

The present experiments were done to determine the effects of repeated exposure to NO_2 during postnatal lung development on the ability of the respiratory tract to clear particulate material that was deposited after the animals had reached young adulthood. A second objective was to determine whether the clearance capability could be recovered upon cessation of the exposures. The ferret was chosen as the animal model because its lung structure in many ways resembles that of the human more than do the lung structures of other species used in toxicologic studies (Phalen and Oldham, 1983; Oldham et al., 1990). Additionally, in a previous study the ferret thoracic clearance pattern was found to be quite similar to that of humans (Mannix et al., 1991). Because the ferret grows very rapidly, from about 15 g at birth to as much as 1 kg at 20 wk, an exposure regimen spanning the period of most rapid lung maturation could be accomplished in a reasonable time frame.

Two NO_2 concentrations were used, with the lower (0.5 ppm) representing a near "worst-case" residential scenario, and a higher concentration (10 ppm) that was known to produce definite lung pathology in other species (e.g., Hackett, 1979; Stephens et al., 1978; Chang et al., 1988) and that we have confirmed in the ferret (Rasmussen, 1992; Rasmussen and McClure, 1992). Daily exposures, begun when the ferrets were 6 wk of age, were divided into 2-h sessions, morning and afternoon, to mimic the episodic exposure that a child might experience during the cooking of meals. The results indicated that the repeated NO_2 exposure reduced the ability of the deep lung to clear small particles, and that, in the case of the higher concentration, the lung did not recover clearance capability during a period of 4 wk with no further exposure. The results suggest that chronic exposure even to low concentrations of pollutants can have measurable, and potentially adverse, effects on vital lung functions. Comparative studies with adult fer-

rets will indicate whether these effects are more pronounced when the exposures occur during the critical period of rapid lung growth.

METHODS

Animals

Pregnant female European domestic ferrets (*Mustela putorius furo*) were obtained from Marshall Farms (North Rose, N.Y.) at approximately 4 wk gestation. The jills had previously been vaccinated against canine distemper and tested negative for Aleutian disease virus. Upon arrival at our laboratory the jills were housed one per cage in an AAALAC-accredited facility until birth of the kits approximately 2 wk later. At weaning (5–6 wk) the kits were segregated by sex and littermates were housed up to four per cage for the duration of the study. Metal ear tags were inserted to ensure identification. Dry food (Purina ferret chow, Purina Mills, St. Louis, Mo., and IAMs Kitten Food, Dayton, Ohio) and water were provided ad libitum. Illumination was on a daily 12 h light–12 h dark cycle, beginning at 7 a.m. Animal handlers wore clean lab coats, surgical masks, disposable vinyl or latex gloves, and hair covers to minimize chances of transmitting infections to this susceptible species (Fox, 1988). Although the ferrets were not housed in air barrier isolators, the room air was HEPA (high efficiency particulate air) filtered and was supplied at 15–20 air changes/h. At the start of exposure (6 wk of age) the mean body mass was 220 g ± 50 g (SD) with no significant difference between the sexes. At 20 wk of age the females weighed 625 ± 136 g, and the males 1092 ± 214 g. These masses are slightly below the published masses for ferrets of this age (McLain et al., 1992). This was attributed to the constant light–dark cycle, inducing a spring-summer response in the weight gain, as contrasted with natural lighting conditions that would induce greater weight gain with shorter periods of daylight in the autumn (McLain et al., 1992). Analysis of the body mass data demonstrated no statistically significant differences (two-tailed t-test; critical $p = .05$) among the exposure groups at either the beginning or end of the experiment.

NO_2 Exposure

The exposure groups consisted of four or five ferrets. Exposures were nose-only, using anodized aluminum manifolds (In-Tox Products, Albuquerque, N. Mex.) designed to prevent rebreathing and cross-breathing between animals. The exposure protocol was meant to mimic the daily time and duration that a child might experience NO_2 exposure when living in a residence where heating and cooking were done with gas or oil burners. Thus, exposures were 2 h each in the morning and afternoon, separated by a 3–4 h break. Three separate groups of ferrets were exposed in this manner to purified air, or to the high (10 ppm) or low (0.5 ppm) concentrations of

NO_2, 5 d/wk for 15 wk. Three additional groups were similarly exposed, but were removed from exposure after 8 wk and were housed in the animal facility without further exposure until the particle clearance measurements began. The purpose for the shorter exposure of these groups was to investigate the possible recovery of clearance function that might occur postexposure.

For exposure the ferrets were restrained in Lexan plastic tubes with anodized aluminum nose cones sealed into the exposure manifold with o-rings. As the ferrets grew during the experiment, the internal diameters of the plastic tubes were increased by using larger tubes or removal of inserts so that the animals were never cramped. Test atmospheres were generated using compressed, dried, and filtered air scrubbed with Purafil (Purafil, Inc., Chamblee, Ga.) and activated charcoal. Particulates were removed with a 0.47-μm pore size glass fiber filter. The airstream was then rehumidified to 55–60% relative humidity, and NO_2 was metered into the air from cylinders of research-grade NO_2 in nitrogen (Matheson Gas Products, Cucamonga, Calif.) to obtain the desired concentrations. Airflow through the system provided at least 1 L/min for each ferret. The system was operated at slight positive pressure (approx. 0.2 in H_2O) to ensure that the animals were exposed to the test atmospheres. The NO_2 concentrations were continuously monitored at the breathing zone of the ferrets (from an unused breathing port) using a Beckman 952A $NO/NO_2/NO_x$ analyzer (Beckman Instruments, Fullerton, Calif.). The instrument calibration was checked daily with a certified NO_2 standard in nitrogen (Scott Environmental, Riverside, Calif.). Variations in concentration during exposure were recorded on a strip-chart recorder and were routinely less than ±5%.

Tracer Particle Deposition and Clearance Measurement

The clearance function of the ferrets was measured over a period of 3 wk beginning at wk 13 of exposure for the 15-wk exposure groups, and, for those ferrets taken off exposure after 8 wk, beginning with wk 5 after the last exposure to NO_2. Clearance was measured by monitoring the disappearance of inhaled radiolabeled particles from the head and thoracic regions as previously described (Mannix et al., 1991). In brief, ^{51}Cr-labeled polystyrene latex microspheres were prepared (Hinrichs et al., 1978) and the ferrets were allowed to inhale an aerosol of the particles for a period of 30 min using a previously described nose-only exposure apparatus (Raabe et al., 1973). The microspheres had an activity median aerodynamic diameter of 1.7 μm with a geometric standard deviation of 1.2 μm, as determined by a Mercer-type impactor (In-Tox Products, Albuquerque, N. Mex.). Immediately after tracer particle deposition, the muzzles of the ferrets were thoroughly washed to remove any external particles. The total radioactivity deposited in the respiratory tract was approximately 1 μCi per ferret. The distribution and quantitation of radioactivity was determined using two collimated sodium iodide scintillation detectors positioned to separately detect radiation from the head and thoracic regions (Mannix et al., 1991). The head airways region

extended from the tip of the nose to the midpoint of the trachea, while the thoracic region included the lower half of the trachea and the lungs. The relatively long trachea of the ferret permits excellent separation of these counting regions. Counting was done while the ferrets were restrained in plastic tubes similar to those used for NO_2 exposure, but without metal nose cones. Each ferret was counted within 15 min of the end of the particle deposition, and subsequently at each of the following time points: 1, 2, 3, 4, 5, 7, 9, 24, 48, 96, 168, 240, 340, and 432 h. The counting time was 100 s up to 24 h, and 200 s for later times. Animals that moved substantially during counting were recounted.

Data Analysis

Graphical representations of the head airways counting data for the groups of ferrets between 0 and 48 h postdeposition indicated that there was generally a very rapid clearance of particles soon after deposition, followed by a period during which the clearance rate was considerably more gradual. Therefore, curve-stripping methodology was employed to separate the data into rapid clearing (0–2.5 h) and slower clearing (4–48 h) components. The counting data for each ferret in each of the two time intervals were subsequently fitted to single exponential functions such that each of the two phases was describable in terms of characteristic slope and intercept values. The double exponential fits of the data were then plotted and compared with the actual observed data—both on an animal-by-animal basis, and for the groups for animals; good agreement was noted as illustrated by the plot of actual data and the calculated curve (Fig. 1). The calculated curve and data points for the ferrets exposed to clean filtered air were essentially the same as in Figure 1. The thoracic region counting data for individual and groups of ferrets between 48 and 432 h postdeposition were adequately fitted by single exponential functions characterizable in terms of a single slope and intercept. The slopes and intercepts for the head airways region and the thoracic region from individual animals were combined in relation to the exposure group, and group statistics were calculated. The results for the groups were compared using an analysis of variance and a two-tailed *t*-test. Two critical values of p were employed: .1 for a basic level of significance, and .05 as a more stringent level of significance.

RESULTS

Head Region Analysis

Since the groups exposed to purified air for 8 wk and for 15 wk had nearly identical clearance patterns, these 2 groups were combined into one 10-ferret control group, which was subsequently statistically compared with the various NO_2-exposed groups. The purified air-exposed group had a clearance pattern very similar to that measured in older control ferrets in a

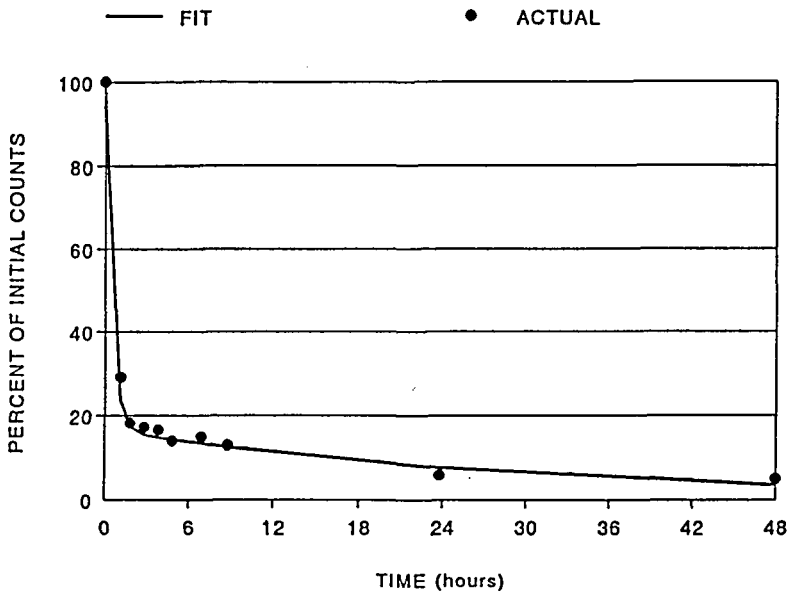

FIGURE 1. Comparison of the calculated double exponential curve fit and the actual head airways region data points for the group of ferrets exposed to 10 ppm NO_2 for 15 wk.

previous study (Mannix et al., 1991). As in the earlier study, about 20% of the tracer radioactivity was presumed to be deposited on slow-clearing epithelium, as evidenced by the time-zero intercept of the slow-clearing component. Previously we shaved the snouts of several ferrets and verified that this phenomenon was not due to external contamination of the fur. Our conclusion is that the particles in question deposited on, or translocated to, nonciliated olfactory epithelium or anterior surfaces in the nasal region, or to regions of damaged and inefficiently clearing epithelium.

In general, neither the 10 ppm nor the 0.5 ppm NO_2 exposures (8 or 15 wk) significantly altered either component of head airways clearance, as compared to the combined control group (Table 1). None of the observed slopes or intercepts were statistically different from control values. In addition, with only one exception, the groups exposed for 15 wk did not exhibit slopes and intercepts that were statistically different from those of the groups exposed to the same concentration for 8 wk and then held for 4 wk until the start of the clearance measurement study. The one exception to this was that the group exposed to 0.5 ppm NO_2 for 8 wk had a significantly different ($p < .1$) long-term component intercept from that of the group exposed for 15 wk. This was surprising, especially since the shorter duration exposed group was the one that deviated more from the control value. We consider this as likely not representing a real effect, but more a statistical anomaly.

Due to the small numbers of ferrets in the exposure groups, it is possi-

ble that significant effects occurred that were not statistically discernible. In order for a real effect to be detected statistically, the mean value would have needed to be about 30–40% different from the control group. Since no duration of exposure effects were noted (with the one exception previously described), the NO_2 exposure groups were combined to statistically evaluate NO_2 concentration-related effects that were independent of length of exposure. Again, no statistically significant effects were noted for any of the parameters studied, demonstrating that the clearance pattern for the head airways region was not greatly affected by the NO_2 exposures.

Thoracic Region Analysis

It was apparent from the raw data that NO_2 exposure tended to delay the long-term thoracic clearance of the tracer particles in a concentration-related manner. A two-tailed *t*-test analysis of the group slope and intercept values (Table 1) demonstrated that both the 8-wk and 15-wk 10 ppm exposure groups exhibited significant ($p < .1$) delays in thoracic clearance. In fact, 3 of the ferrets in the 10 ppm NO_2 groups experienced what appeared to be complete deep lung clearance shutdown (slope = 0.00000%/h). Ferrets in both the 8-wk and 15-wk groups were thus affected, demonstrating the persistence of the NO_2-mediated effect. Although the clearance rates for the two 0.5 ppm NO_2 groups were also reduced from control values, these effects were not statistically significant. None of the intercept values from the NO_2-exposed groups were significantly different from the control intercept, nor were there any significant differences for either the 10 ppm or 0.5 ppm groups between the slope and intercept values for the 8-wk and 15-wk groups (i.e., no duration of exposure or damage-repair effects).

TABLE 1. Group Clearance Data

NO_2 group (ppm)	*n*	Head airways region				Thoracic region	
		Short-term component		Long-term component			
		Intercept[a]	Slope[b]	Intercept[a]	Slope[b]	Intercept[a]	Slope[b]
0	10	73.27 (8.23)	1.66 (0.29)	20.0 (10.9)	0.031 (0.007)	100.5 (5.0)	0.00066 (0.00026)
0.5[c]	5	71.32 (7.01)	1.67 (0.33)	13.2 (3.3)	0.028 (0.003)	105.9 (4.9)	0.00056 (0.00044)
0.5	5	77.48 (2.80)	1.63 (0.28)	18.0 (3.5)	0.036 (0.008)	101.4 (5.4)	0.00054 (0.00042)
10[c]	4	73.61 (5.82)	1.45 (0.3)	22.8 (3.7)	0.039 (0.007)	99.4 (6.0)	0.00018[d] (0.00021)
10	5	77.71 (5.48)	2.12 (1.03)	16.7 (7.8)	0.032 (0.014)	102.7 (8.5)	0.00031[e] (0.00036)

Note. Values are means (SD).

[a]Intercept expressed as percent of first count for head airway region and percent of 48-h count for thoracic region.

[b]Slope expressed as the fraction of remaining activity cleared per hour (isotope decay corrected).

[c]Exposed for first 8 wk of study and not exposed during or after clearance experiment.

[d]Significant at $p < .05$.

[e]Significant at $p < .1$.

Morphometric analyses (measurements of alveolar dimensions, cellularity, total lung collagen, lesions, and lung size; Rasmussen and McClure, 1992) performed postmortem revealed that the effects observed in the NO_2 groups exposed for 15 wk were not statistically significantly different from the effects noted in the groups exposed for 8 wk and then held for 4 wk before the start of the clearance study. In other words, the deep lung effects, as judged from histopathology, were also quite persistent. For this reason, and because (a) the ferret groups sizes were small and (b) the measured thoracic clearance slopes for ferret groups exposed to the same NO_2 concentrations were consistent with regard to degree and direction from control, it was deemed appropriate to combine the group data (10 ppm NO_2, 8-wk and 15-wk groups combined, etc.) for statistical comparison. When this was done the delay in clearance experienced by the combined 10 ppm NO_2 group was very significant ($p < .05$). The combined 0.5 ppm NO_2 group slope, although less than the control, was not statistically significantly different from that of the combined control group.

DISCUSSION

Several epidemiologic studies have suggested that indoor air pollution is associated with increased respiratory disease in children (reviewed by Angle, 1988, and Samet, 1991). Candidate causative agents are NO_2, secondhand tobacco smoke (Samet et al., 1991), and "dampness," which presumably would encourage growth of molds and bacteria (Brunekreef et al., 1989). A limited number of reliable measurements of indoor NO_2 concentrations (Samet and Spengler, 1989; Samet et al., 1992; Leaderer, 1982) have suggested that levels approaching 1 ppm may be reached under adverse conditions. Outdoor concentrations rarely approach these levels, but can rise during episodes of temperature inversions and high pollution (California Air Resources Board, 1991). Therefore, the potential for repeated exposure of children to significant concentrations of NO_2 exists both indoors and in the urban environment.

The exposure schedule used in the present study was based on a scenario of daily periods of high indoor levels of NO_2 associated with cooking with a gas stove in a poorly ventilated room. Additional NO_2 might be contributed from smokers and from heating appliances. The lower concentration (0.5 ppm) might reasonably be expected to occur in such situations, but not the higher concentration of 10 ppm. However, high concentrations of NO_2 are encountered in certain situations in industry and farming. It is worth noting that the permissible exposure level for occupationally exposed humans, determined as an 8-h time-weighted average (TWA), was at one time 5 ppm. Even a single 4-h exposure to the latter concentration is clearly damaging to the lung of the young ferret (Rasmussen, 1992).

The clearance data reported here are in good agreement with that previously reported for the normal young adult ferret (Mannix et al., 1991), and

are distinctly different from the clearance patterns of rodents as reported by others (Bailey et al., 1989) and measured in our laboratory using the same methods as in the present work (Kenoyer et al., 1981; Mannix et al., 1982). Therefore, it is of limited value to compare directly the slopes found for particle clearance from the head and thoracic regions among the various species. Also, the various investigators have used particles of differing chemical composition and size, which adds to the difficulty of comparison among species. In general, it seems that exposures to concentrations of NO_2 that produce limited irritation tend to have no effect or to accelerate clearance, as the result, presumably, of increased mucus production (e.g., Ferin and Leach, 1977; Vollmuth et al., 1986; Wolff, 1986). Chronic exposure to concentrations of NO_2 above 5 ppm, where histopathologic effects are produced or direct effects on alveolar macrophages occur (Davis et al., 1992), may inhibit clearance. However, large differences have been found among species in both the direction of the effect on the clearance function and the effective concentration of NO_2 (see review by Schlesinger, 1992), so that no true generalizations can be made across species lines.

The mechanism for the observed reduction in long-term particle clearance from the ferret lung cannot be specified at this time. The principal route of particle clearance from the alveolar region is considered to be engulfment by alveolar macrophages, followed by transport of the loaded macrophage up the mucociliary ladder, and elimination via the gut (Wolff, 1986; Lehnert, 1992). In studies with the mouse, Davis et al. (1992) found that exposure to 10 ppm NO_2 had a direct effect on the viability of pulmonary macrophages obtained by bronchoalveolar lavage, but for macrophages recovered at 7 d postexposure, the viability and ability to kill engulfed bacteria were normal. This suggests that our results are not due to a direct lethal effect on macrophages, since long-term clearance remained depressed at 4 wk after the end of exposure to 10 ppm NO_2. Alternative routes of macrophage-associated clearance are via the interstitium and/or lymphatic system, leading to more or less permanent deposition of material in the thoracic regional lymph nodes (Lehnert, 1992). We have reported increased collagen deposition in the lungs of the same ferrets exposed to 10 ppm NO_2 in the present clearance study (Rasmussen and McClure, 1992). Quantitative histologic examination has indicated an up to threefold increase in connective tissue in the submucosa of the respiratory bronchiolar epithelium (Rasmussen et al., 1993), suggesting that clearance via the interstitium surrounding the respiratory bronchioles and the proximal alveolar walls may be inhibited. A third possibility is that the cellular composition or morphology of the terminal bronchiolar epithelium may have been modified in a way that reduced the ability of particle-containing macrophages to reach the mucociliary ladder. Chang et al. (1992) in studies with the rat chronically exposed to low concentrations of ozone found persistent changes including increased interstitial fibrosis, and evidence of toxic effects on ciliated and Clara cells in the terminal bronchioles. This

loss of cilia would be expected to affect movement of the mucus layer and clearance of particulate material from the alveolar region, which has been observed after exposure of rats to ozone (Wolff, 1986). We have not yet determined whether similar alterations may be present in the respiratory bronchioles of ferrets exposed to NO_2.

It has not been definitely established that long-term exposure of young humans to air pollution has permanent adverse effects in the lung. However, studies of lungs of trauma victims in the Los Angeles basin suggest a high prevalence of chronic tissue inflammation in the region of the terminal and respiratory bronchioles (Sherwin, 1991). From this observation it can be reasonably extrapolated that similar damage occurs in the lungs of young children living in the same area. Since children tend also to be relatively active, any effects of air pollution would likely be amplified (Silverman et al., 1976; Mautz et al., 1985).

The results presented in this paper, together with previous work from this laboratory (Rasmussen and McClure, 1992; Kenoyer et al., 1981) and others (e.g., Schlesinger et al., 1987; Wolff, 1986), indicate that subchronic or even episodic exposure to pollutants can lead to deficits in certain lung defense mechanisms, and to structural changes in the lung that might compromise lung gas exchange functions. Further work is required to establish dose-response relationships in exposures that include exercise as a variable, and atmospheres that contain representative concentrations of the other major pollutants.

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