

Urinary Porphyrin Profiles as Biomarkers of Trace Metal Exposure and Toxicity: Studies on Urinary Porphyrin Excretion Patterns in Rats during Prolonged Exposure to Methyl Mercury

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Urinary Porphyrin Profiles as Biomarkers of Trace Metal Exposure and Toxicity: Studies on Urinary Porphyrin Excretion Patterns in Rats during Prolonged Exposure to Methyl Mercury. WOODS, J. S., BOWERS, M. A., AND DAVIS, H. A. (1991). *Toxicol. Appl. Pharmacol.* **110**, 464-476. Studies were conducted to define the specific changes in the urinary porphyrin excretion pattern (porphyrin profile) and the time course of those changes in rats exposed to mercury as methyl mercury hydroxide (MMH) at 5 or 10 ppm in the drinking water for up to 30 weeks. The urinary porphyrin profile elicited by MMH is uniquely characterized by highly elevated levels of 4- and 5-carboxyl porphyrins, and of a third atypical porphyrin with as yet undetermined chemical characteristics. Changes in the porphyrin profile were observed as early as 1 or 2 weeks following initiation of exposure to MMH at 10 or 5 ppm, respectively, and were sustained as long as 40 weeks following cessation of MMH treatment. The magnitude of the urinary porphyrin profile at either MMH dose level increased progressively during the course of mercury treatment and was highly correlated with the renal mercury concentration. A subsequent decline in the magnitude of the urinary porphyrin profile in animals exposed to 10 ppm MMH for more than 10 weeks was associated with the accumulation of high levels of Hg²⁺ in kidney cells and loss of renal functional status. These findings demonstrate that mercury elicits a unique change in the urinary porphyrin excretion pattern which is related to the dose and duration of mercury treatment. The association of urinary porphyrin excretion rates with renal mercury content and functional status suggests that urinary porphyrin profiles may serve as a useful biomarker of mercury accumulation and nephrotoxicity during prolonged mercury exposure. © 1991 Academic Press, Inc.

Porphyrins are formed as intermediates in the biosynthesis of heme (Fig. 1), a process which proceeds in essentially all eukaryotic tissues. In humans and other mammals, porphyrins with 8, 7, 6, 5, and 4 carboxyl groups are commonly produced in excess of that required for heme synthesis and are excreted in the urine in a well-established pattern. Previous studies from this laboratory have described metal-specific changes in urinary porphyrin excretion patterns (porphyrin profiles) during prolonged exposure of animals to low levels of mercury (Woods and Fowler, 1977), arsenic (Woods

and Fowler, 1978), and lead (Fowler *et al.*, 1980) compounds. The etiology of these changes has been shown to involve both the metal-induced impairment of specific heme biosynthetic pathway enzymes in target tissues, as well as metal-facilitated oxidation of reduced porphyrins which accumulate in tissue cells because of impaired porphyrin metabolism (Woods *et al.*, 1990). Changes in porphyrin excretion patterns associated with metal exposures are largely metal-specific and occur prior to the onset of target tissue damage as assessed by biochemical or ultrastructural techniques. These observations have suggested the potential utility of porphyrin profile measurements as biomonitors of metal exposure

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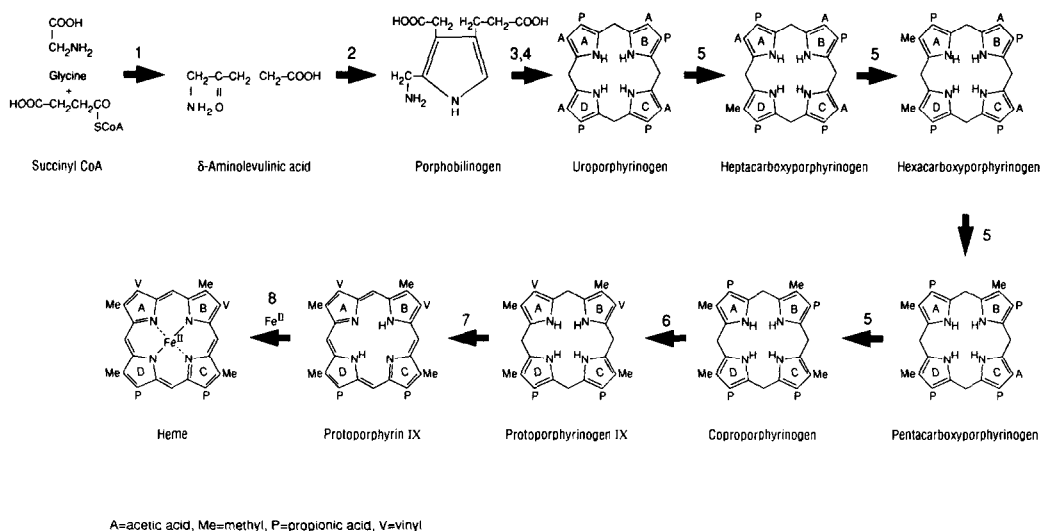


FIG. 1. Heme biosynthetic pathway. Steps are catalyzed by (1) δ -aminolevulinic acid (ALA) synthetase, (2) ALA dehydratase, (3) uroporphyrinogen I synthetase, (4) uroporphyrinogen III cosynthetase, (5) uroporphyrinogen decarboxylase, (6) coproporphyrinogen oxidase, (7) protoporphyrinogen oxidase, and (8) ferrochelatase.

and biological effects in human subjects (Woods, 1989; Marks, 1988; Fowler *et al.*, 1987).

Of particular interest to the investigation of metal-induced porphyriurias are findings from studies of methyl mercury-exposed rats which have demonstrated a dramatic change in the urinary porphyrin excretion pattern due primarily to mercury-induced alterations of heme biosynthesis in the kidney (Woods and Fowler, 1977; Woods *et al.*, 1984). Inasmuch as the kidney is a principal target organ of mercury compounds, these observations suggest that changes in urinary porphyrin excretion patterns might serve as a specific measure of the accumulation and biological effects of mercury in the kidney during prolonged metal exposure.

The present studies were undertaken to define the specific changes in the urinary porphyrin excretion pattern elicited during prolonged exposure to mercury as methyl mercury hydroxide (MMH) in rats, and to describe the relationship of porphyrinogenic changes to renal mercury content. The findings demonstrate that the mercury-specific porphyrin profile reflects both dose- and time-dependent

effects of mercury in the kidney and, hence, might serve as a useful biomarker of mercury concentration and effects over a prolonged course of mercury exposure.

MATERIALS AND METHODS

Materials. Male Fischer-344 rats (150–175 g) were obtained from Simonsen Labs (Gilroy, CA). Analytichem C-18 Bond Elut large reservoir capacity (LRC) 10-ml disposable columns were acquired from Varian (Harbor City, CA). Porphyrin standards of the I isomeric configuration were obtained from Porphyrin Products (Logan, UT). Methyl mercury hydroxide was purchased from Alfa Products (Danvers, MA). Methanol was HPLC Grade. All other chemicals were reagent grade and were obtained from standard commercial sources. Distilled deionized water was used for all aqueous solutions.

Prolonged exposure study. Immediately upon receipt from the supplier, 90 rats were transferred to individual hanging, wire-bottom cages and permitted access to food (Wayne Rodent Blox) and deionized water *ad libitum*. The diet was carefully evaluated prior to the study with respect to the content of metals or other potentially porphyrinogenic substances and found to contain no materials capable of interfering with porphyrin metabolism or assessment. Animals facilities were maintained at $22 \pm 1^\circ\text{C}$ and on a 12-hr light/dark cycle. Following a 1-week acclimation period at these conditions, all animals were placed in individual metabolism cages with continuous

access to water, and 24-hr urine collections were made for baseline urinary porphyrin determinations. Rats were then divided into three groups of 30 animals each and continued either on deionized water (controls) or deionized water containing 5 or 10 ppm MMH, respectively. These dosages were selected from previous studies (Woods and Fowler, 1977) as moderately and more severely nephrotoxic, respectively, in prolonged exposure regimens. MMH treatments were continued for up to 30 weeks with water consumptions recorded daily. At weekly intervals following the commencement of MMH exposure, 24-hr urine collections were made from 6 rats from each dose group for porphyrin and mercury analyses. Immediately following urine collections, 2 animals from each dose group were euthanized for kidney mercury analysis and other assessments. The remaining 4 animals in each dose group were returned to their respective exposure regimen. Throughout the course of the prolonged exposure study, urine was collected from the same 4 animals per group at weekly intervals, along with the 2 additional animals per group to be euthanized for biochemical evaluations. This longitudinal design provided a measure of the consistency of urinary porphyrin concentrations in the same animals from week to week throughout the course of the study, while also permitting independent cross-sectional evaluation of parameters of interest on a weekly basis. Necropsy of animals that died during the course of MMH exposure was performed within 24 hr of death by the University's Department of Comparative Medicine.

Postexposure study. Prior to the initiation of the 90-rat, 30-week study described above, a separate prolonged exposure study was undertaken to determine the extent to which mercury-induced changes in the porphyrin excretion pattern would persist after cessation of exposure of rats to MMH. In this study, 12 rats were divided into two treatment groups receiving deionized water (controls) or water containing 10 ppm MMH, respectively. MMH treatment continued for 8 weeks, during which time 24-hr urinary porphyrin measurements in all 12 animals were made at weekly intervals according to the regimen described above. After 8 weeks, the 6 rats receiving MMH were transferred to deionized water, and the urinary porphyrin concentrations of all 12 animals were monitored for a subsequent 40 weeks.

Collection of urine samples. For 24-hr urine collections, rats were placed in individual metabolism cages provided with water or water containing MMH. To avoid photochemical oxidation of porphyrins, urines were collected in foil-wrapped 125-ml polypropylene flasks containing 50 mg NaHCO_3 (to maintain neutral pH) and 4 mg EDTA (to prevent potential metal complexation of porphyrins). Following collection, total urine volume was measured and either processed immediately for porphyrin analysis or frozen at -80°C .

Urinary porphyrin analysis. Rat urine differs from that of humans in containing relatively high concentrations of protein as well as riboflavin and other fluorescing materials which might potentially interfere with the spectrofluores-

cent detection of specific urinary porphyrins. To deal with these differences, we have developed an analytical procedure which improves on previously employed methods (Schreiber *et al.*, 1983; Woods and Southern, 1989) by permitting separation of potentially interfering urinary contaminants as well as by providing for improved porphyrin recovery. Following collection, urine samples are centrifuged at $2,000g$ for 5 min to remove excess NaHCO_3 and any insoluble materials. Three to 5 ml of the supernatant is then acidified to pH 2 with 6 N HCl and is transferred into a 10-ml disposable C-18 Bond Elut column which has been pretreated, first with 10 ml of 100% HPLC grade methanol followed by 10 ml of 10 mM phosphate buffer, pH 3.5. Flow through the column is initiated by applying slight vacuum, facilitated by use of a vacuum system such as the Baker 10 Extraction System (Baker Chemicals Co., Phillipsburg, NJ) employed in this lab. The sample is allowed to gravity drip through the column until flow ceases. Porphyrins are retained on the column, while a major fluorescent contaminant, including riboflavin, passes through and is discarded. The column (to which the porphyrins are bound) is then sequentially washed first with 10 ml 10 mM phosphate buffer, pH 3.5, then with 40 ml of 35% methanol in 10 mM phosphate buffer, pH 3.5 (to remove remaining interfering contaminants); then with 3 ml of 10 mM phosphate buffer, pH 3.5; and finally, with 10 ml of 10 mM phosphate buffer, pH 7.5. The final wash adjusts the column pH for elution of porphyrins. The preceding four washing steps may be facilitated by use of a vacuum system such as that described above. Porphyrins are eluted from the column by first passing 1 ml of 100% methanol followed by 2 ml of 80% methanol in 10 mM phosphate buffer, pH 7.5, through the column. The collected porphyrins are concentrated by evaporating off the eluant under a stream of N_2 at $40\text{--}60^\circ\text{C}$ and are then reconstituted in 0.5 ml 1 N HCl. A 50- μl aliquot is used for HPLC analysis, conducted as previously described (Woods *et al.*, 1984). Porphyrin fluorescence is monitored at an emission wavelength of 620 nm following excitation at 395 nm. Recovery of porphyrin standards by this method is 87 to 100% in HCl or when added to rat or human urine in concentrations from 3 to 67 pmol/ml.

Tissue mercury analysis. Mercury concentrations in kidney cortex, urine, and blood were determined using a continuous flow, cold vapor atomic absorption system recently developed in this department based on procedures described by Oda and Ingle (1981). Total mercury, as well as Hg^{2+} species, were directly quantitated. CH_3Hg^+ concentrations were computed as the difference between total mercury and Hg^{2+} . This method permits highly reproducible recoveries from spiked tissues in excess of 95% for both inorganic and methyl mercury species. Quantitation limits for inorganic mercury and for total mercury in tissues were 0.3 and 0.4 $\mu\text{g}/\text{gm}$, respectively. The details of this procedure are currently in preparation for publication.

Other assays. Protein concentrations were determined by the method of Smith *et al.* (1985) using the bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, IL).

Statistical analyses. Analysis of significance of differences between treatment groups was determined by Student's *t* test. The level of significance was chosen at $p < 0.05$. Linear regression analyses were performed using a SYSTAT general purpose statistics package acquired from SYSTAT, Inc. (Evanston, IL).

RESULTS

Water consumption was consistent among all animals over the 20- to 30-week study period. Average water consumption was 30 ± 5 ml/rat/day. MMH dosage in the 5 and 10 ppm treatment groups was approximately 0.75 and 1.5 mg/kg/day, respectively.

Initial studies were conducted to characterize the specific changes in the urinary porphyrin profile elicited during prolonged MMH exposure. Figure 2 describes the HPLC porphyrin elution profiles from 24-hr urine samples of control rats (Fig. 2A) and of rats exposed continuously to 10 ppm MMH for 5 weeks (Fig. 2B). To facilitate comparison, data are presented as the logarithms of the concentrations of each porphyrin. The principal response to MMH treatment is a significant elevation in the concentration of porphyrins with five or fewer carboxyl groups. Coproporphyrin (4-carboxylporphyrin) was elevated to 10.5 times control levels in the 10 ppm MMH treatment group after 5 weeks of exposure from 1439 ± 180 to $15192 \pm 1,629$ pmol/24 hr (means \pm SD). Pentacarboxylporphyrin was elevated 8.2-fold from 39 ± 7 to 320 ± 83 pmol/24 hr. Additionally, a porphyrin with as yet unknown chemical characteristics which elutes approximately midway between 5- and 4-carboxyl porphyrins on HPLC (precoproporphyrin) was observed in MMH treated rats at a concentration approximately equivalent to that of pentacarboxylporphyrin. Porphyrins with 8, 7, and 6 carboxyl groups were not significantly elevated in urine of MMH-treated rats. Animals exposed to 5 ppm MMH for 5 weeks displayed a urinary porphyrin profile with comparable characteristics, although the magnitude of the change in 4- and 5-carboxyl porphyrins, as well as in precoproporphyrin, was substantially less than that observed in 10 ppm exposed rats.

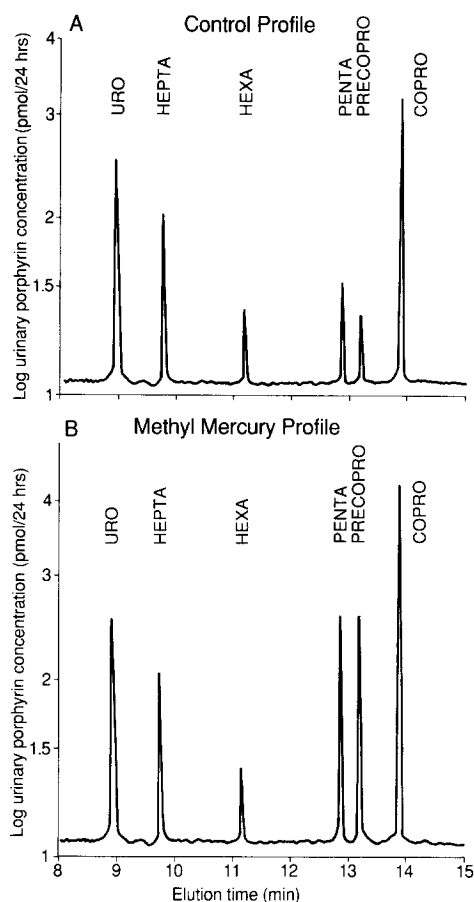


FIG. 2. HPLC elution profiles of urinary porphyrins from unexposed Fischer rats (A) or from rats exposed to methyl mercury hydroxide (MMH) (B) at 10 ppm for 5 weeks. Data are presented as the \log_{10} of the mean porphyrin concentration in 24-hr urine samples of six individual animals. Porphyrin concentrations in this and subsequent figures were determined as described under Materials and Methods. Porphyrin concentrations (pmol/24 hr, means \pm SD) of untreated (control) rats were: uro, 355 ± 81 ; hepta, 104 ± 71 ; hexa, 22 ± 8 ; penta, 39 ± 7 ; copro, 1439 ± 180 . Porphyrin concentrations of MMH-treated rats were: uro, 362 ± 96 ; hepta, 117 ± 72 ; hexa, 21 ± 10 ; penta, $320 \pm 83^*$; copro, $15192 \pm 1629^*$. *Significantly different from control value ($p < 0.05$).

The time course of the development of the urinary porphyrin profile during prolonged MMH treatment was studied in rats exposed to 5 or 10 ppm MMH over a period of 30 or 20 weeks, respectively. The time-related changes in urinary porphyrin concentrations in the 10 ppm treatment group are described

in Fig. 3. During exposure to 10 ppm MMH, the mean urinary coproporphyrin concentration (Fig. 3A) was significantly increased to nearly two times control levels as early as 1 week following initiation of MMH exposure and continued to increase progressively throughout the first 5 weeks of treatment. From Weeks 5 to 6, the urinary coproporphyrin concentration plateaued, but then again increased substantially to a maximum of 30 times control levels ($36,963 \pm 6577$ MMH versus 1265 ± 152 pmol/24 hr, controls) between the 6th and 9th weeks of exposure. Coproporphyrin levels then declined gradually over the subsequent 10 weeks of continuing MMH exposure to approximately three times control levels.

A similar pattern was observed with respect to pentacarboxylporphyrin (Fig. 3B) and precoproporphyrin (Fig. 3C) in 10 ppm MMH-exposed animals. The maximum increase in the mean urinary concentration of pentacarboxylporphyrin was 1404 ± 130 pmol/24 hr, 17.5 times control levels after 10 weeks of MMH exposure. The concentration of pentacarboxylporphyrin then declined over the ensuing 13 weeks but remained elevated at 3.2 times control levels after 20 weeks of 10 ppm MMH treatment. Precoproporphyrin followed a similar pattern over the course of MMH exposure.

Body weights of the four animals initially assigned to the 10 ppm MMH exposure group for longitudinal porphyrin assessment increased comparably to those of controls (from 178 ± 31 to 239 ± 49 g) throughout the initial 10 weeks of MMH treatment, and no gross anatomical or physical abnormalities were seen. During the subsequent 10 to 20 weeks of MMH exposure, however, body weights declined progressively, with two of the animals developing hind limb paresis after 15 weeks of MMH exposure. One animal succumbed after 18 weeks and another after 19 weeks of MMH treatment. The others were euthanized following the 20th week of porphyrin analysis, after we concluded that they would not likely survive continuing MMH treatment. Necropsy findings from these animals confirmed

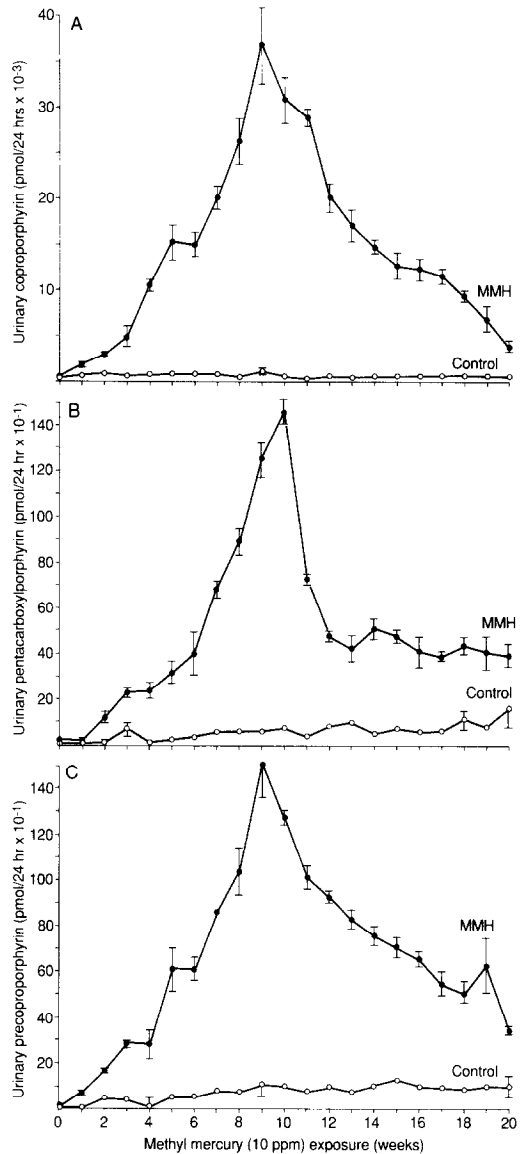


FIG. 3. Time course of changes in urinary coproporphyrin (A), pentacarboxylporphyrin (B), and precoproporphyrin (C) during exposure to MMH at 10 ppm. Values represent means \pm SEM of six separate determinations at each time point. All values in MMH treated rats except for zero time determinations and pentacarboxylporphyrin at Week 1 are significantly different ($p < 0.05$) from control values. Ranges of the SEM for points without error bars are contained within the data points.

death due to loss of renal function characterized by chronic interstitial nephritis and glomerular degeneration.

The time course of development of the mercury-specific urinary porphyrin profile in rats given 5 ppm MMH was characterized by a similar course of progressive increase in the concentrations of pentacarboxyl-, precopro- and coproporphyrins during the initial phase of the exposure period (Fig. 4). However, the increase of each porphyrin occurred at a slower rate than observed in the 10 ppm treatment group. Moreover, a progressive declining phase

in urinary porphyrin concentrations, such as that observed in rats treated with MMH at 10 ppm for 9 weeks or longer, was not seen in 5 ppm MMH-exposed rats. As shown in Fig. 4A, a significant increase in the urinary coproporphyrin concentration was observed within 2 weeks of MMH treatment at 5 ppm, but the magnitude of change was substantially less than that observed in the 10 ppm treated animals (Fig. 3A). Urinary coproporphyrin in-

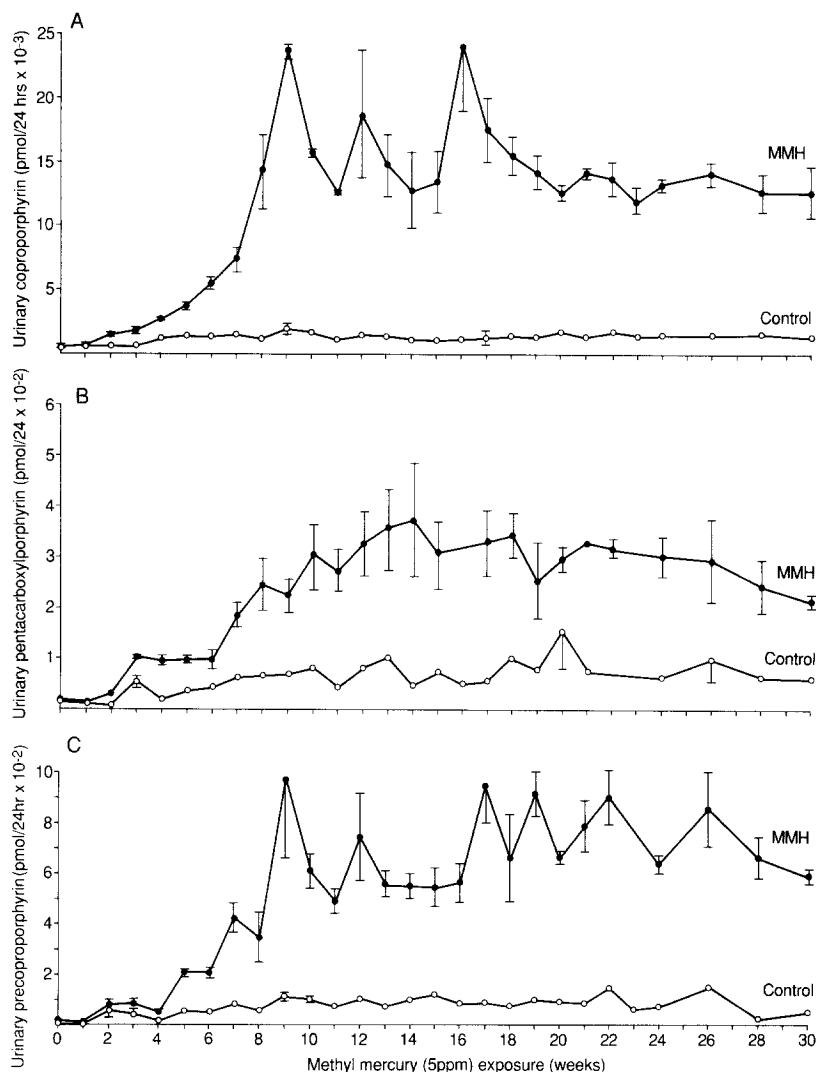


FIG. 4. Time course of changes in urinary coproporphyrin (A), pentacarboxylporphyrin (B), and precoproporphyrin (C) during exposure to MMH at 5 ppm. Values represent means \pm SEM of six separate determinations at each time point. All values in MMH treated rats except for zero time and Week 1 determinations and for precoproporphyrin at Week 2 are significantly different ($p < 0.05$) from control values. Ranges of the SEM for points without error bars are contained within the data points.

creased progressively throughout the first 9 weeks of 5 ppm MMH treatment, reaching a maximal concentration of $23,683 \pm 602$ pmol/24 hr, approximately 15 times that measured in unexposed rats (1539 ± 154 pmol/24 hr). Between Weeks 9 and 14, urinary coproporphyrin levels fluctuated between 10 and 15 times those of controls then remained relatively constant at approximately 10 times control values ($13,190 \pm 564$ pmol/24 hr) throughout the ensuing 14 weeks of exposure to MMH at 5 ppm. Comparable patterns of progressive increase followed by maintenance of relatively constant levels of the urinary concentrations were observed with respect to pentacarboxylporphyrin (Fig. 3B) and precoproporphyrin (Fig. 3C) over the prolonged course of exposure of rats to MMH at 5 ppm.

Studies were conducted to compare the development of the porphyrin profile with changes in renal mercury content during the course of prolonged MMH exposure. Changes in renal mercury concentration during treatment of rats with MMH at 10 ppm are shown in Table 1. The concentration of total mercury in kidney cortex increased progressively over the initial 10 weeks of 10 ppm MMH treatment. Total renal Hg content reached approximately $140 \mu\text{g/g}$ wet wt ($0.70 \mu\text{mol/g}$) at 10 weeks of treatment and then declined over the subsequent 9 weeks to a mean concentration of $107 \mu\text{g/g}$ ($0.53 \mu\text{mol/g}$). Speciation analysis of renal mercury content revealed that the concentrations of both organic (CH_3Hg^+) and inorganic (Hg^{2+}) increased continuously over the initial 10-week portion of the exposure period. However, the proportion of total mercury present as CH_3Hg^+ then declined during subsequent exposure, while the proportion of total mercury present as Hg^{2+} continued to increase. The balance between organic and inorganic mercury species in kidney cortex appeared to remain relatively constant between the 3rd and 10th weeks of 10 ppm MMH exposure, with approximately equal concentrations of each species sustained over this period. Subsequently, however, the concentration of inorganic mercury increased to as much as 80% of total mercury in renal cor-

TABLE 1
CHANGES IN RAT RENAL CORTICAL MERCURY CONTENT DURING PROLONGED EXPOSURE TO METHYL MERCURY HYDROXIDE (MMH) AT 10 ppm IN DRINKING WATER

Weeks exposure	Renal mercury ($\mu\text{g/g}$)		Total Hg
	Hg^{2+}	CH_3Hg^+	
0	0	0	0
1	3.9 (26)	10.8 (74)	14.7
2	14.6 (38)	23.4 (61)	38.0
3	28.5 (49)	29.6 (51)	58.1
5	46.4 (50)	46.4 (50)	92.8
6	62.8 (52)	55.9 (48)	118.7
7	68.0 (54)	56.0 (46)	124.0
9	70.8 (55)	58.0 (45)	128.8
10	78.5 (56)	61.6 (44)	140.1
11	78.0 (57.5)	57.7 (42.5)	135.7
14	80.3 (68.3)	37.3 (31.7)	117.6
19	85.7 (79.9)	21.6 (20.1)	107.25

Note. Kidneys were acquired from two rats at times indicated during treatment with MMH at 10 ppm. Values represent the means of individual determinations. Numbers in parentheses are the percentage of total mercury present as organic or inorganic species. Mercury assays were conducted as described under Materials and Methods.

tex, while the organic constituent decreased markedly.

Comparison of the time course of development of the urinary porphyrin profile in 10 ppm MMH-exposed rats with changes in renal mercury content demonstrates that urinary porphyrin concentrations increase concomitantly with both organic and inorganic mercury species during the initial 10 weeks of MMH exposure. As shown in Fig. 5, the time course of increase in urinary coproporphyrin levels appears to correspond closely with the period during which the ratio of organic to inorganic mercury species is ≤ 1 in kidney tissue. In contrast, the decline in urinary porphyrin levels during the subsequent 10 weeks of MMH exposure (Weeks 10 to 20) corresponds to a marked increase in the renal concentration of the inorganic species. The onset of the declining phase of the porphyrin profile in 10 ppm MMH-exposed rats corresponds to

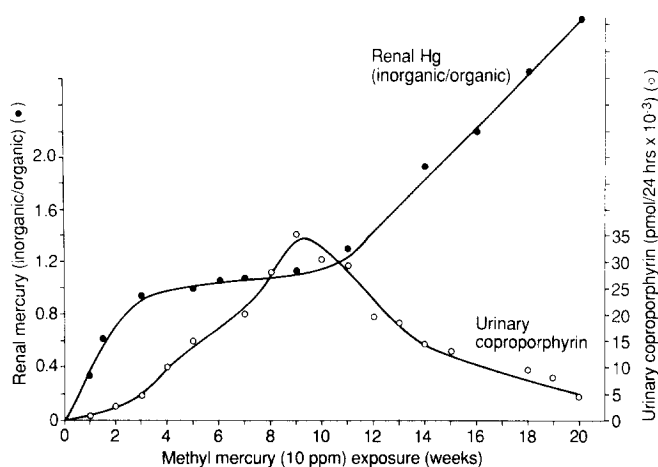


FIG. 5. Time course of changes in renal mercury content and urinary coproporphyrin concentration during 10 ppm MMH exposure. Mercury data are expressed as the ratio of inorganic to organic mercury species in renal tissue. Mercury and porphyrin determinations were made as described under Materials and Methods.

a renal Hg^{2+} concentration in excess of $70 \mu\text{g/g}$ ($0.35 \mu\text{mol/g}$).

Table 2 presents the changes in total renal mercury content, as well as in organic and inorganic species, during prolonged exposure of rats to MMH at the more moderate 5 ppm dose level. The total mercury content of the kidney increased throughout the initial 14 weeks of exposure, although at a slower rate than observed in 10 ppm exposed animals. The balance between organic and inorganic mercury species again seemed to remain relatively constant between the 3rd and 9th weeks of 5 ppm MMH exposure. Subsequently, the renal concentration of organic mercury declined while inorganic mercury increased, although at a much more gradual rate than observed in 10 ppm treated rats.

Comparison of the time course of development of the urinary porphyrin profile with changes in renal mercury concentrations in 5 ppm MMH-exposed rats again demonstrates that the increase in urinary porphyrin concentrations corresponds to the period during which the ratio of organic to inorganic mercury species remains at ≤ 1 in the kidney. As shown in Fig. 6, the magnitude of the urinary porphyrin profile, as reflected in the urinary coproporphyrin concentration, increased pro-

gressively over the initial 9 weeks of MMH exposure, during a concomitant increase in renal mercury content. During ensuing exposure (Weeks 10–20), the magnitude of the porphyrin profile remained relatively constant or declined slowly, consistent with the gradual increase in inorganic mercury species as a proportion of total renal mercury content.

Regression analyses demonstrated a highly significant correlation between urinary porphyrin concentrations and renal mercury content at each dose level during the period of increasing renal mercury content. As shown in Table 3, correlation coefficients (r^2) for each regression suggest a close association of each of the three elevated porphyrins with renal mercury as organic or inorganic species or as total mercury content.

Changes in urinary mercury concentrations during the course of MMH exposure reflected those occurring in the kidney at each dose level. As shown in Table 4, organic mercury constituted 79% of total mercury in the urine of rats exposed to MMH at 10 ppm for 1 week. The proportion of mercury as organic species decreased progressively throughout the exposure period, although approximately equivalent concentrations organic and inorganic species were maintained after 6 weeks of

TABLE 2

CHANGES IN RAT RENAL CORTICAL MERCURY CONTENT DURING PROLONGED EXPOSURE TO METHYL MERCURY HYDROXIDE (MMH) AT 5 ppm IN DRINKING WATER

Weeks exposure	Renal mercury ($\mu\text{g/g}$)		
	Hg ⁺²	CH ₃ Hg ⁺	Total Hg
0	0	0	0
1	2.3 (36)	4.1 (64)	6.4
2	6.0 (42)	8.3 (58)	14.3
3	9.6 (46.4)	11.1 (53.6)	20.7
4	15.0 (46.4)	17.3 (53.6)	32.3
5	18.7 (46.4)	21.7 (53.6)	40.4
6	27.0 (49)	27.95 (51)	54.95
9	53.0 (52.5)	47.95 (47.5)	100.9
11	60.44 (54.5)	50.46 (45.5)	110.9
14	67.4 (56.4)	52.15 (43.6)	119.55
19	65.1 (62.5)	39.05 (37.5)	104.15

Note. Kidneys were acquired from two rats at times indicated during treatment with MMH at 5 ppm. Values represent the means of individual determinations. Numbers in parentheses are the percentage of total mercury present as organic or inorganic species. Mercury assays were conducted as described under Materials and Methods.

MMH treatment. A similar pattern was observed among rats exposed to MMH at 5 ppm (data not shown). In rats given 10 ppm MMH a significant increase in the magnitude of the urinary porphyrin profile was apparent after 1 week of MMH treatment (Fig. 3), corre-

TABLE 3

STATISTICAL ASSOCIATION OF URINARY PORPHYRIN CONCENTRATIONS WITH RENAL MERCURY CONTENT DURING PROLONGED MMH EXPOSURE

MMH exposure	Porphyrin	Correlation coefficient (r^2)		
		Total Hg	CH ₃ Hg ⁺	Hg ⁺²
5 ppm	Copro	0.724	0.745	0.683
	Pentacarboxyl	0.782	0.811	0.744
	Precopro	0.857	0.872	0.850
10 ppm	Copro	0.934	0.909	0.872
	Pentacarboxyl	0.844	0.988	0.970
	Precopro	0.943	0.990	0.903

Note. Linear regression analyses were performed using the SYSTAT general purpose biostatistics package (SYSTAT Inc.). Correlation coefficients (r^2) denote the proportion of variance in the levels of porphyrins accounted for by total mercury or mercury species. The 5 ppm data were for Exposure Weeks 0 to 14; 10 ppm data were for Exposure Weeks 0 to 8.

sponding to urinary organic and inorganic mercury concentrations of 0.11 $\mu\text{g/ml}$ (0.55 nmol/ml) and 0.03 $\mu\text{g/ml}$ (0.15 nmol/ml), respectively.

In the separate postexposure study described under Materials and Methods, rats were exposed either to distilled water or to water containing 10 ppm MMH for a period of 8 weeks. Porphyrin profiles were monitored during exposure and subsequently at periodic intervals for up to 40 weeks following cessation of

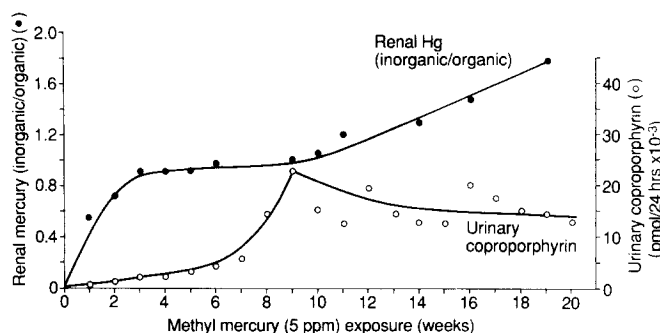


FIG. 6. Time course of changes in renal mercury content and urinary coproporphyrin concentration during 5 ppm MMH exposure. Mercury data are expressed as the ratio of inorganic to organic mercury species in renal tissue. Mercury and porphyrin determinations were made as described under Materials and Methods.

TABLE 4

CHANGES IN RAT URINARY MERCURY CONCENTRATIONS DURING PROLONGED EXPOSURE TO METHYL MERCURY HYDROXIDE (MMH) AT 10 ppm IN DRINKING WATER

Weeks exposure	Urinary mercury ($\mu\text{g}/\text{ml}$)		
	Hg^{+2}	CH_3Hg^+	Total Hg
0	0	0	0
1	0.03 (21)	0.11 (79)	0.14
2	0.08 (15)	0.47 (85)	0.55
6	0.48 (49)	0.49 (51)	0.97
8	0.675 (49)	0.70 (51)	1.375
9	1.26 (52)	1.185 (48)	2.445
11	0.85 (55)	0.70 (45)	1.55
15	0.63 (56)	0.50 (44)	1.13
17	0.593 (57)	0.447 (43)	1.04

Note. Urine was collected from two rats at the times indicated. Values represent the mean mercury concentrations of pooled samples. Numbers in parentheses represent the percentage of total urinary mercury present as inorganic or organic species. Mercury determinations were made as described under Materials and Methods.

TABLE 5

CHANGES IN URINARY COPROPORPHYRIN CONCENTRATIONS FOLLOWING CESSATION OF MERCURY EXPOSURE

Assessment period	Urinary coproporphyrin	
	($\text{pmol}/24 \text{ hr}$)	Percentage control
Preexposure	1,441 \pm 191	101
8 weeks at 10 ppm MMH	25,284 \pm 4227	1413
1 week postexposure	18,194 \pm 2056	960
2 weeks postexposure	15,006 \pm 3129	799
3 weeks postexposure	9,850 \pm 2306	730
5 weeks postexposure	6,743 \pm 405	405
20 weeks postexposure	2,109 \pm 287	180
30 weeks postexposure	2,066 \pm 434	165
40 weeks postexposure	1,534 \pm 198	152

Note. Twenty-four-hour urine samples were collected from six rats at times indicated before, during, and subsequent to MMH exposure at 10 ppm in drinking water. Values in MMH-treated rats are compared with those of controls measured at the same times. Values represent means \pm SEM of separate determinations. Urinary porphyrin levels were determined as described under Materials and Methods.

TABLE 6

DISTRIBUTION OF INORGANIC AND ORGANIC MERCURY SPECIES IN KIDNEY AND BLOOD AFTER 19 WEEKS OF MMH TREATMENT AT 10 ppm

Tissue	Mercury concentration ($\mu\text{g}/\text{g}$)		
	Hg^{+2}	CH_3Hg^+	Total Hg
Kidney	72.5 (82)	16.0 (12)	88.5
Blood	6.4 (14)	40.0 (86)	46.4

Note. Kidneys were acquired from two rats exposed to MMH at 10 ppm in drinking water for 19 weeks. Blood was acquired from the same animals by cardiac puncture. Data represent results of pooled kidney and blood assessments. Numbers in parentheses represent the percentage of total mercury present as inorganic or organic species. Mercury assays were conducted as described under Materials and Methods.

MMH treatment to evaluate the relationship between porphyrin excretion and renal mercury clearance. The results, presented in Table 5, show that the urinary coproporphyrin concentration declined rapidly following cessation of MMH exposure, but remained elevated at 1.5 times control levels up to 40 weeks after exposure was terminated. Urinary pentacarboxyl- and precoproporphyrins followed a similar pattern, but were not significantly different from control levels after 20 weeks following cessation of MMH treatment. Total renal mercury content declined from 125 $\mu\text{g}/\text{g}$ after 8 weeks of MMH treatment to 4.7 $\mu\text{g}/\text{g}$ at 40 weeks post MMH exposure.

Finally, studies were conducted to demonstrate that speciation of MMH occurred within the kidney per se, rather than extrarenally with subsequent uptake of organic and inorganic species by kidney cells. Table 6 shows the distribution of organic and inorganic mercury in blood and kidney in rats after 19 weeks of MMH treatment at 10 ppm. The data indicate that, while organic mercury constituted only 12% of total renal mercury content, 86% of mercury in the blood remained in the organic form.

DISCUSSION

Numerous studies have proposed the potential utility of monitoring urinary porphyrin

excretion patterns as biomarkers of chemical exposure and effects in target tissues (Marks, 1988; Woods, 1989; Fowler *et al.*, 1987; Woods and Fowler, 1978). The present study defines the characteristics of porphyrin profile changes elicited by mercury as MMH during prolonged, low-level exposure in rats and describes the association of these changes with alterations in kidney and urinary mercury content.

Of particular interest from these studies is the observation that mercury exposure elicits a specific change in the porphyrin excretion pattern unlike that reported of any other known porphyrinogenic substance. This pattern, characterized by significantly increased urinary concentrations of coproporphyrin, pentacarboxyl porphyrin, and of a porphyrin (precoproporphyrin) which elutes on HPLC intermediate between the former two, is sustained throughout the course of prolonged exposure to MMH at variable dose levels, as well as up to at least 40 weeks following cessation of MMH treatment. These observations suggest the potential utility of porphyrin profile measurements as a specific indicator of mercury exposure.

The time course of change in the magnitude of the porphyrin profile during prolonged MMH treatment is closely associated with the tissue concentration of inorganic mercury species (Hg^{2+}), suggesting that the biological effects underlying the development of the porphyrin profile are mediated by Hg^{2+} . This concept is consistent with findings from this study that organic mercury is converted by renal cells *in vivo* to the Hg^{2+} form, as well as with previous observations from this laboratory (Woods and Southern, 1989; Woods *et al.*, 1984; Woods, 1989) which demonstrate the direct inhibitory action of Hg^{2+} on coproporphyrinogen oxidase as well as other enzymes of the kidney heme biosynthetic pathway. Should the porphyrinogenic response to MMH be mediated by the Hg^{2+} form *in vivo*, as suggested from the present study, these findings would suggest that urinary porphyrin profiles would be of value in monitoring ex-

posure to mercury in either organic or inorganic forms.

Of additional interest is the observation that the conversion of organic mercury to the inorganic form in the kidney occurs in a dose- and time-dependent manner during prolonged MMH exposure, with approximately equal concentrations of organic and inorganic species attained when the total mercury concentration reaches approximately 60 $\mu\text{g/g}$ (0.3 $\mu\text{mol/g}$) of kidney tissue. This concentration was attained in the 5 and 10 ppm MMH exposure groups after 6 and 3 weeks of MMH exposure, respectively. The kidney appears to be capable of maintaining this balance between organic and inorganic species over a relatively wide range of total mercury levels, inasmuch as approximately equal concentrations of both species were found in either treatment group at total renal cortical mercury concentrations up to approximately 120 $\mu\text{g/g}$ (0.6 $\mu\text{mol/g}$), twice that at which this balance was initially achieved. The increasing phase of the urinary porphyrin profile in both 5 and 10 ppm MMH treatment groups occurs over the period during which equal concentrations of organic and inorganic species are achieved and sustained, whereas the declining phase in the 10 ppm treatment group corresponds to loss of ability by the kidney to maintain this balance in the face of increasing tissue Hg^{2+} content. Thus, to the extent that the maintenance of approximately equal concentrations of organic and inorganic mercury species during MMH exposure reflects functional renal capacity, the porphyrinogenic response as manifested by the magnitude of the urinary porphyrin profile appears to represent functional kidney status.

The decline in the overall magnitude of the porphyrin profile which is observed in 10 ppm MMH-exposed animals after 9 or 10 weeks of MMH treatment (Weeks 10–20) (Fig. 3) occurs concomitantly with a decline in the total renal mercury content, but, with a sustained increase in renal Hg^{2+} levels. Previous biochemical and ultrastructural studies demonstrate a dramatic decline in renal function as well as a loss of structural integrity of mito-

chondria and other subcellular constituents during the latter stage of prolonged mercury exposure (Kacew and Hirsch, 1981; Chen *et al.*, 1983; Fowler and Woods, 1977; Fowler, 1972). An increased level of oxidative tissue damage and lipid peroxidation is also observed during this time. The *rate* of increase in total renal mercury content declines rapidly during this latter phase of the prolonged exposure period, suggesting that a decrease in the rate of incorporation of MMH by renal proximal tubule cells accounts for the decline in tissue CH_3Hg^+ content observed. The concomitant increase in Hg^{2+} concentrations may, therefore, reflect the oxidative demethylation of CH_3Hg^+ already present in kidney cells, a process perhaps anticipated in the face of the extensive mercury-induced oxidative tissue damage observed.

The plateau phase in the increase in urinary porphyrin concentrations observed between the 5th and 6th weeks of treatment in the 10 ppm MMH-exposed group (Fig. 3) may reflect an adaptive response of the kidney to mercury exposure, such as induction of metallothionein or other mercury-binding protein (Goyer, 1984). This effect could reasonably explain the hiatus in the porphyrinogenic action of mercury, inasmuch as induction of metallothionein is known to occur as a delayed response to metal exposure (Singhal *et al.*, 1987). The decline in the magnitude of the urinary porphyrin profile observed between Weeks 9 and 11 in the 5 ppm MMH treatment group may represent a comparable response of the kidney to mercury exposure, since this effect occurs at comparable tissue Hg^{2+} concentrations (approximately $50 \mu\text{g/g}$) at which the porphyrin plateau was observed in the 10 ppm treatment group.

The chemical nature and etiology of the precoproporphyrin that elutes between pentacarboxyl- and coproporphyrins in mercury-treated rats are as yet unknown, although preliminary spectral studies on the isolated material confirm that it is a porphyrin species. Studies with pentacarboxyl- and coproporphyrin standards of the III isomeric configuration demonstrate it is not a stereoisomer of

either of these porphyrins. The possibility that it is a chemical isomer of coproporphyrin is suggested by studies of Elder (1972, 1974) which demonstrated the synthesis of isocoproporphyrins in liver of hexachlorobenzene-treated rats under conditions predisposing to the preferential metabolism of pentacarboxylporphyrinogen, rather than coproporphyrinogen, by coproporphyrinogen oxidase. Such conditions could arise in the present studies if pentacarboxylporphyrinogen accumulates in sufficient concentrations in kidney cells during MMH exposure to successfully compete with coproporphyrinogen as the enzyme substrate. Further studies are in progress with the isolated precoproporphyrin to determine its chemical characteristics and its biochemical etiology.

In conclusion, the present studies demonstrate that prolonged exposure of Fischer rats to mercury as MMH elicits a unique change in the urinary porphyrin excretion pattern which may be specific to mercury exposure. The time course of the change in the magnitude of the urinary porphyrin profile corresponds to increasing concentrations of mercury in the kidney cortex and may reflect the functional adaptability of the kidney to mercury over a wide range of mercury concentrations. The declining phase of the urinary porphyrin profile, observed only in the 10 ppm exposure group, reflects the loss of functional renal capacity and overt toxicity in the face of increasing tissue mercury content. These results suggest the predictive and diagnostic potential of urinary porphyrin profiles as a biomarker of mercury exposure and nephrotoxicity.

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