Journal of Toxicology and Environmental Health, Part A Current Issues

ISSN: 0098-4108 (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/uteh19

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To cite this article: Deadre J. Johnson , Leon Lack , Sherif Ibrahim , Susan M. Abdel-Rahman & Mohamed B. Abou-Donia (1995) Protein-bound pyrroles in rat hair following subchronic intraperitoneal injections of 2,5-hexanedione, Journal of Toxicology and Environmental Health, Part A Current Issues, 45:3, 313-324, DOI: 10.1080/15287399509531998

To link to this article: https://doi.org/10.1080/15287399509531998

Published online: 19 Oct 2009.

PROTEIN-BOUND PYRROLES IN RAT HAIR FOLLOWING SUBCHRONIC INTRAPERITONEAL INJECTIONS OF 2,5-HEXANEDIONE

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Studies were initiated to ascertain whether body hair could be used to develop a biological marker for chronic exposure to industrial neurotoxicants that yield the metabolite 2,5-hexanedione (2,5-HD), that is, n-hexane and methyl n-butyl ketone. Rats were injected daily with a 50 mg/kg ip dose of 2,5-HD for 45 d. At intervals, body hair and individual vibrissae were removed (under general anesthesia) and tested for the presence of pyrrole substances with p-N,N-dimethylaminobenzaldehyde (DMAB, Ehrlich's reagent). Vibrissae and body hair were stained a reddish color that was distinctly different from that observed with the hair taken from control animals. Solubilized body hair protein from the treated animals gave a positive Ehrlich's test, while that from control animals was negative. Spectral analysis of the DMAB-treated hair from experimental animals disclosed a maximum absorbance at 530 nm, which indicated the presence of pyrrole substituents. Serial analysis of individual nose hairs taken during 2,5-HD administration showed a progression with time of the region staining positively for pyrroles, thus indicating that the process can proceed in growing hair. These findings suggest the potential utility of hair as an indicator for chronic exposure to this class of industrial chemicals possessing neurotoxicity potential. This could complement urinary analysis, which is now used to confirm recent exposure.

Neurotoxicity evoked by the industrial solvents *n*-hexane and methyl *n*-butyl ketone is believed to be caused by their common metabolite, 2,5-hexanedione (2,5-HD) (Spencer et al., 1975; Abou-Donia et al., 1982, 1985a, 1985b, 1991). 2,5-HD is capable of forming N-substituted pyrroles by reacting with primary amines. This includes amino acids as well as the ϵ-amino groups of lysine-containing peptides (DeCaprio, 1986; DeCaprio et al., 1982; Graham et al., 1982). In vivo accumulation of intracellular protein-bound pyrroles during chronic administration of 2,5-HD will represent the result of a number of factors: uptake of 2,5-HD by the specific cells, formation of pyrrolated polypeptides, oxidative alterations of these pyrrole-like

Received 8 April 1994; accepted 11 October 1994.

This work was supported by NIOSH grant OHO-0823. The authors acknowledge Susan Reeves and Steven Conlon for their skillful preparation of the photomicrographs and Shirley Faulkner for her excellent secretarial assistance. Leon Lack received support for this work from the Research Grant Committee of Duke University Medical Center.

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Journal of Toxicology and Environmental Health, 45:313–324, 1995

0098-4108/95 $10.00 + .00

substances, and clearance/turnover by the cells, which very likely involves the removal of peptides bearing oxidized or altered pyrrole adducts, as well as unaltered pyrrole adducts. DeCaprio et al. (1983) studied the effect of 2,5-HD neurotoxicity in chickens. Their examination of the time course of pyrrole tissue levels during daily dosing demonstrated early pyrrole peaks followed by declines of detectable protein-bound pyrroles, despite continuing administration of neurotoxin.

Rats would appear to respond differently to such chronic treatment. Protein-bound pyrrole levels in serum and brain stem appeared to reach a plateau in 2 wk. The spinal cord levels plateaued somewhat later (DeCaprio & O'Neill, 1985). These variations probably reflect the complexity of factors listed earlier.

Our interest in exploring the possibility of utilizing the formation of pyrroles as the basis of developing a biological marker for ongoing exposure to these neurotoxins led to the consideration of hair as the tissue for analysis. The pilary system has certain characteristics that make it attractive for this purpose:

1. The material is readily accessible, affording no difficulty of providing the requisite amounts for analysis.
2. Biological turnover (prior to shedding) of the cell contents is minimal. Most of the proteins and DNA of the formed hair are not degraded, and hair can be used as a record of varying metabolic and toxicological states of the individual.
3. With hair, one can distinguish early biological signals from later events. This becomes a particularly important property for a biomarker designed to assess the overall time of chronic exposure to a toxic substance (see the Discussion section).

Before initiating any program directed toward the perfecting of such a pilary-based biomarker, certain prerequisite facts must be established:

1. Can follicular hail bulbs, in fact, take up systemically circulating 2,5-HD?
2. Can this 2,5-HD be incorporated into the intracellular proteins of newly formed hair cells as pyrroles?
3. Do these intracellular protein-bound pyrroles demonstrate sufficient long-term chemical stability that could allow them to serve as a basis for a long-term biomarker?
4. Does this ongoing process of 2,5-HD uptake and protein alteration still permit hair growth to continue?
5. Can soluble proteins be prepared from hair taken from 2,5-HD-treated animals that respond positively for the presence of pyrroles when tested with the Ehrlich's reagent?

This article describes our experimental findings, which answer the preceding conditional questions positively. In addition, we report how these

findings compared with the simultaneous daily urinary excretion of pyrrole-like substances, which Kessler et al. (1990) demonstrated appear in the urine of rats, as well as humans, following their exposure to either 2,5-HD or *n*-hexane.

MATERIALS AND METHODS

Chemicals

2,5-Hexanedione, 2,5-dimethylpyrrole, sodium nitroferricyanide, and *p*-*N*,*N*-dimethylaminobenzaldehyde (DMAB) were obtained from Aldrich (Milwaukee, Wis.). *N*-Ethylmaleimide (NEM) was obtained from Sigma Chemical Company (St. Louis, Mo.).

2,5-Hexanedione and 2,5-dimethylpyrrole were redistilled in vacuo, made up in single-use aliquots, and stored at –20°C and –80°C, respectively. DMAB was stored in separate amber bottles under argon.

Animals

Male Sprague-Dawley rats were supplied by the Charles River Laboratories (Raleigh, N.C.). Initial weights ranged between 280 and 320 g. Animals were housed in individual metabolic cages (Nalge Company, Rochester, N.Y.) that allowed for timed-fluid collections. Food and water were provided ad libitum. Diurnal lighting was maintained at 12-h cycles.

Treatments

Four rats were injected ip with a 50 mg/kg dose of 2,5-HD in 0.5 ml aqueous solution. Injections were made at 5 p.m. Urine was collected at 16 and 24 h following injection. Control urines were obtained for each rat for 3 d prior to the initiation of treatment. Urines were stored at –20°C prior to their analysis. Under these conditions, the pyrrole-like urinary substances have been reported to be stable (Kessler et al., 1990).

Chemical Determination of Pyrrole-Like Substances in the Urine

We modified DeCaprio's method (DeCaprio et al., 1982) by adding sufficient acid to counteract the buffering capacity of the urine. In addition, the specified amounts of NEM were added to prevent possible inhibition by any sulfhydryl groups that may have been present, since it has been reported that sulfhydryl groups interfere with Ehrlich's reaction (Gibson et al., 1955). Preliminary experiments established that NEM does not affect the assay. DeCaprio et al. (1982) also stated that their conditions for this assay were such that they avoided interference from those naturally occurring substances that would be expected to respond positively to Ehrlich's test.

Samples of urine were filtered through 0.45-μm filters prior to analysis. Aliquots of 10, 20, and 40 μl of filtered urine were adjusted as needed to 40 μl with distilled water and were then tested simultaneously. To these were added 2.6 ml of 1% sodium dodecyl sulfate (SDS); 0.2 ml 50% aqueous

methanol containing 1% concentrated HCl; 0.2 ml 1% NEM in methanol; and 0.4 ml 1.5% DMAB in 50% methanol containing 1% concentrated HCl. The reactants in test tubes were mixed and kept at room temperature for 1 h. All samples were read against reagent blanks containing 40 μl of distilled water at 525 nm in a Shimadzu UV 3000 spectrophotometer. Nonspecific absorption at 525 nm was determined by employing control urines (described earlier). Linearity at 525 nm was always observed with these sample volumes. Parallel determinations were made with 2,5-dimethylpyrrole standard freshly prepared as 10 μg/ml in 50% ethanol–water.

Analysis of Rat Hair

Samples of body hair, as well as individual vibrissa, were plucked from the animals while they were anesthetized with diethyl ether. The sites of hair removal, dorsal region and flanks, were distant from the sites of injection. Body hair was placed in test tubes to which the following chemicals were added: 1 ml methanol containing 50 mg NEM, 2 ml of 1% SDS in water, and 2 ml of 2.7% DMAB in 50% alcohol–water containing 1% concentrated HCl. Samples were allowed to sit overnight at room temperature away from light (in stoppered test tubes). After 15–18 h, the fluid was removed and the samples were washed twice with 50% methanol–water with 1% concentrated HCl, twice with methanol, three times with chloroform, once with methanol, and once with ethanol. Samples were then allowed to air dry at room temperature in absence of light.

The vibrissae were allowed to react overnight submerged in a solution of 2.7% DMAB in 50% ethanol–water containing 1% concentrated HCl. Fluid was removed and the vibrissae washed as follows: twice with 50% methanol-water containing 1% concentrated HCl, twice with methanol, once with chloroform, and twice with ethanol. The samples were allowed to air dry in the absence of light.

Preparation of Soluble Hair Proteins

The procedures employed were those described by Gillespie (1991). They involve fission of the disulfide bonds with β-mercaptoethanol in 8 *M* urea, pH 10.5–11, followed by centrifugation. Solubilized proteins in the supernatant were treated with iodoacetic acid to alkylate the sulfhydryl groups. This was followed by extensive dialysis. In order to protect against autoxidation of intracellular pyrroles during these procedures, we carried out these steps under argon.

Immunofluorescent Studies of Rat Skin (Frozen Sections)

Polyclonal antibodies against protein-bound pyrroles were obtained by inoculating rabbits with pyrrole-derivatized bovine serum albumin (BSA-pyrrole). Pyrrole derivatization of BSA was carried out by the procedures described by DeCaprio (1987). The hapten was prepared by incubating the carrier molecule, BSA, with 2,5-HD in phosphate buffer, pH 9.5, in the

presence of 0.1% sodium azide at a ratio of 50 molecules of 2,5-HD to each lysine group in the BSA. The reaction was run under nitrogen at 37°C for 24 h. The reaction mixture was dialyzed against sodium phosphate buffer, pH 9.5, containing 0.05% sodium azide under nitrogen for 12 h. Dialysis was carried out for another 24 h after changing the dialysis buffer. The product was stored in 50-μl aliquots under nitrogen at –80°C. The purity and identity of the product was determined using SDS polyacrylamide gel electrophoresis (PAGE). 2,5-HD-treated BSA demonstrated a single spot just below the reference spot for BSA (parallel lanes). No protein comparable to BSA was discernible in the lanes containing protein that had been exposed to 2,5-HD. The spot stained positively for pyrrole-bound protein by Ehrlich's reagent, while reference BSA was negative, indicating that the product was the BSA-pyrrole derivative. This was similar to that reported by DeCaprio et al. (1987).

Rabbits were immunized with subcutaneous injections of 60 μg BSA-pyrrole derivative in Freund's adjuvant. The animals were boosted monthly for 4 mo followed by bleeding; 100-μl aliquots of sera were stored under nitrogen at –80°C.

The technique of indirect immunofluorescence was used to stain 4-μm frozen sections of rat skin using the polyclonal antibody already mentioned at a dilution of 1 : 20. Fluorescein-conjugated goat anti-rabbit polyclonal immunoglobulin G (IgG; Sigma Chemical Co., St. Louis, Mo.) was used as a secondary reagent.

RESULTS

Figure 1 shows hair from four rats taken at three different time periods and treated with the Ehrlich's reagent. The top row is hair taken prior to receiving 2,5-HD. The middle row is hair taken from each animal after receiving daily injections of 2,5-HD (50 mg/kg) for 45 d, and the bottom row is hair taken from each rat 25 d after the cessation of 2,5-HD administration. Each hair sample was removed from body areas distal to the site of injection. Note that the hair taken as controls prior to 2,5-HD, as well as the control vibrissae (see later), stained yellow when treated with the DMAB Ehrlich's reagent. These findings for our controls are in agreement with DeCaprio et al. (1983), who also reported the same color response to this reagent by the control tissues that they studied. Hair samples from the rats following 2,5-HD administration were significantly different from controls. This would include those body hair samples that were taken 25 d after cessation of 2,5-HD administration. This persistent reddish-brown color suggests the presence of additional chromophore(s), which would indicate the red color demonstrating the presence of pyrrole groups.

To substantiate this finding, solubilized extracts of hair proteins from these animals were treated with the Ehrlich's reagent. Only the proteins from the 2,5-HD-treated animals gave positive Ehrlich's tests. Spectral analysis of

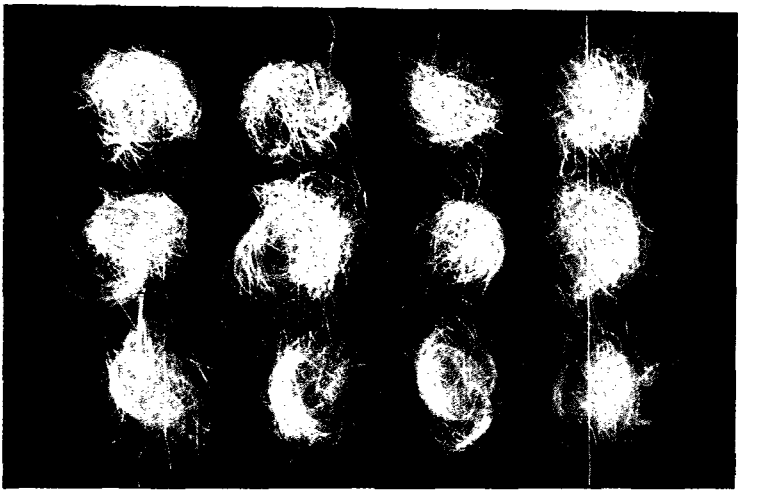

FIGURE 1. DMAB-treated body hair samples from four control rats and four experimental animals. The top row shows hair samples from control rats. The middle row is body hair taken from 2,5-HD-treated rats after receiving 45 daily injections of 50 mg/kg (ip) of 2,5-HD. The bottom row represents body hair samples taken from the experimental animals 25 d after the cessation of 2,5-HD administration. Magnification 1×. Reprinted with permission from Johnson et al. (1994).

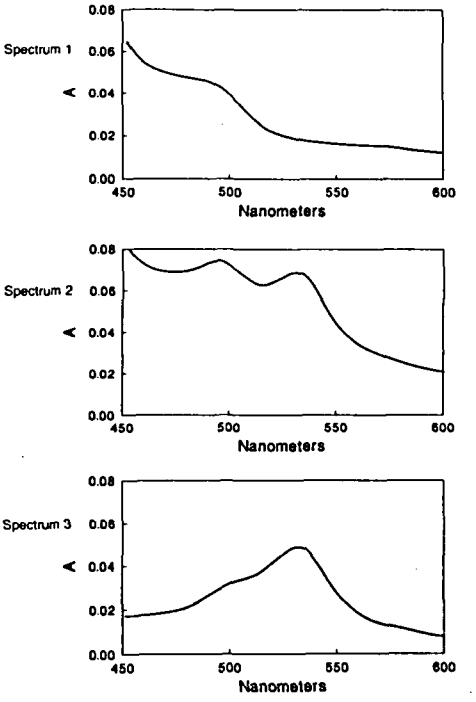

FIGURE 2. Absorption spectra of solubilized body hair proteins from control and 2,5-HD treated rats (50 mg/kg/d for 45 d) following their reaction with DMAB. Spectrum 1, control hair read against reagent blank; spectrum 2, hair protein from treated rats against the reagent blank; spectrum 3, "difference" spectrum between 1 and 2. Each cuvette had 1 ml of protein solution containing 3.1 mg protein, 1.2 ml 1% SDS, 0.1 ml 1% NEM in methanol, and 0.2 ml 1.5% DMAB in 50% methanol–water with 1% HCl. The reagent blanks had the protein solutions replaced with water.

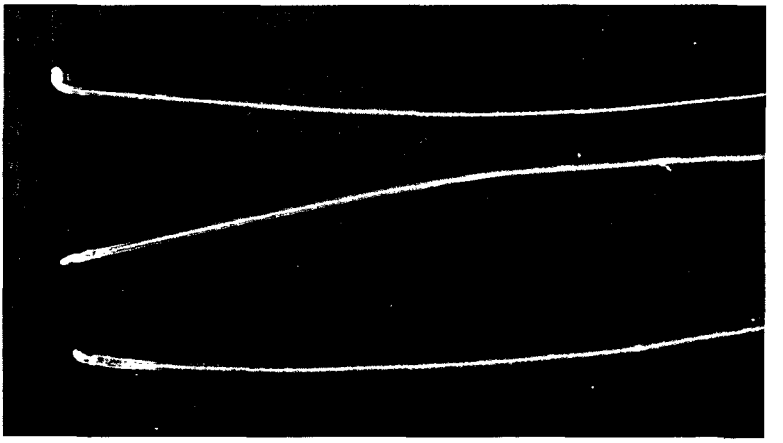

FIGURE 3. Individual vibrissae taken from a rat at different times during its 45-d treatment with 2,5-HD (50 mg/kg/d) and treated with DMAB. From top to bottom, 0 wk, 2 wk, and 5 wk; magnification 1.5x; follicle ends are on the left.

these preparations are shown in Figure 2. Spectrum 1 was obtained when protein solutions from control rats were analyzed with the Ehrlich's reagent. The spectrum was read against the appropriate reagent blank. Spectrum 2 was obtained from protein solutions from 2,5-HD-treated rats; reagent blank again served as the reference cuvette. Spectrum 3 was obtained when the sample cuvette from spectrum 2 was read against that from spectrum 1, that is, a "difference" spectrum. The absorbance maximum at 530 nm seen in spectrum 2 becomes better defined when the nonspecific absorbance is compensated for by using DMAB-treated control protein as a blank. Thus the resulting red color observed when both the hair samples and solutions of solubilized proteins taken from the treated rats were analyzed with the Ehrlich's reagent is due to the absorbance maximum at 530 nm, which is in the range characteristic of this type of pyrrole. 2,5-Dimethylpyrrole, following reaction with DMAB, developed the typical red color whose absorbance spectrum was similar to that of spectrum 3. The absorbance maximum in this instance was 528 nm. This result is similar to that reported by others (DeCaprio, 1986) and is not shown.

Figure 3 shows the vibrissae from a representative animal that received daily ip doses of 50 mg/kg 2,5-HD. Vibrissae were taken at time 0, 2 wk, and 5 wk after the beginning of 2,5-HD injections. The progressive increase in the regions that stained positively for pyrrole adducts demonstrates a causal relationship between the length of the stained area and the number of days the animals received the neurotoxicant. Initiation of the stained areas took place at the follicle end of the hair shafts. This demonstration with single hairs shows the ongoing temporal nature of the positive DMAB response to ongoing administration of 2,5-HD.

Figures 4A and B depict our findings from the immunological studies of

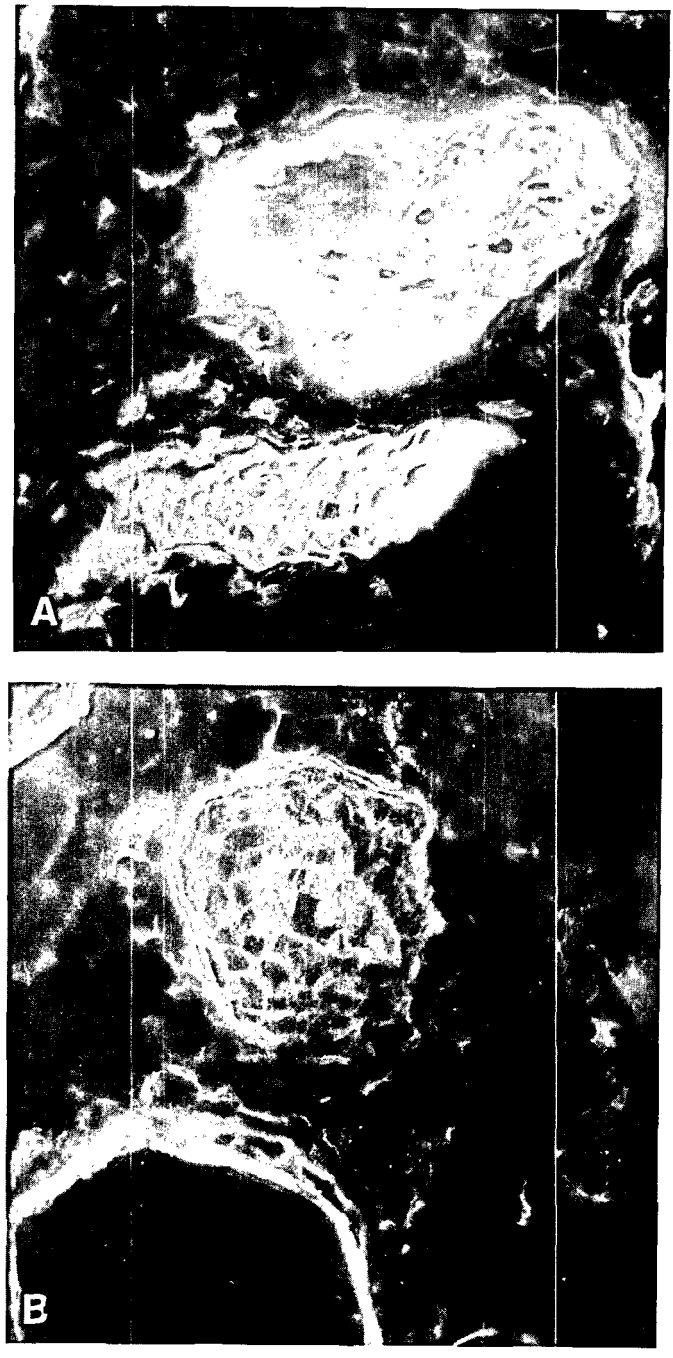

FIGURE 4. Photomicrographs of frozen sections of skin of (A) 2,5-HD treated animal and (B) control animal stained by the antisera to pyrrole-derived BSA as described in the Methods section. Magnification 400x.

frozen sections taken from control rats and treated rats on d 40 of 2,5-HD administration. These microphotographs of cross sections of hair bulbs indicate significantly greater staining of the bulbs of treated animals compared to those of controls.

Figure 5 depicts the average daily output of urinary pyrrole-like substances from the four rats described earlier. Each composite bar shows the average of the daily output of pyrrole-like substances of an individual rat for the 45-d period. As can be seen, 96% of each daily pyrrole excretion took place during the first 16 h following 2,5-HD administration, indicating rapid elimination. Evaluations of the ongoing daily urine excretion of these pyrrolic substances over the 45-d period showed no significant increases or decreases with time of the daily urinary excretion of pyrroles, as indicated by the Pearson correlation coefficients (*r*). The *r* values for rats 1, 2, 3, and 4 were .161, .257, .561, and .583, respectively.

DISCUSSION

The foregoing experimental results stemmed from our investigations directed toward ultimately developing a biological marker based on hair tissue, which would allow evaluation of chronic exposure to those neurotoxi-

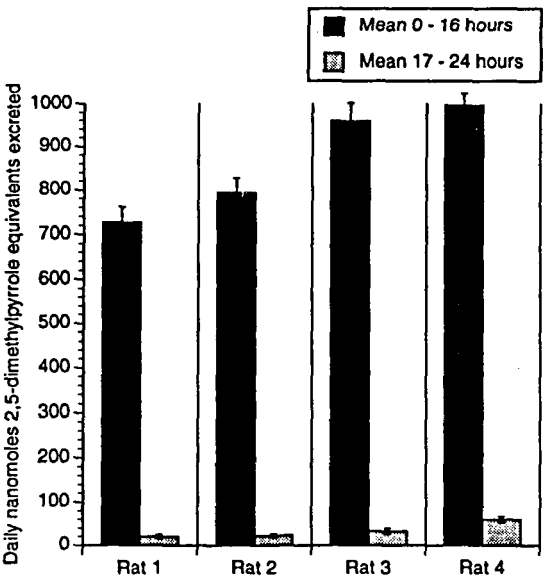

FIGURE 5. Individual daily output averaged (±SEM) over a 45-d period of urinary pyrrole-like substances (expressed as 2,5-dimethylpyrrole equivalents) from four rats that were treated with 50 mg/kg/d 2,5-HD for the 45-d period. The 45-d average individual output would be the sum of the 0–16 h and the 17–24 h collections. Each double bar represents the output from an individual rat.

cants that can be metabolized to 2,5-HD (Lapadula et al., 1986, 1988, 1991). This diketone would form pyrrole adducts with proteins, whose presence in tissues could be detected and quantified with the DMAB reagent. The advantages of utilizing hair for this purpose have already been listed in the introductory section. One of the more interesting ones is that hair allows for the possible development of a method that could assess the duration of chronic exposure and would be independent of the dosages that occurred during this period of ongoing exposure.

However, certain heretofore unreported biological interactions of 2,5-HD with the pilary system must, as critical prerequisites, be ascertained before directly undertaking efforts for establishing such a method. These also have been alluded to in the introduction. They include determining whether 2,5-HD is taken up by the pilary process and whether this does result in the elaboration of protein-bound pyrroles in the mature hair fibers. It was also critical to ascertain that these pyrroles are of sufficient stability to allow them to be utilized for chronic exposure studies. In addition, one would have to be certain that the advent of 2,5-HD to the hair elaboration system does not in fact interfere with hair growth. Finally, the anticipated method would require that the process of preparing soluble hair proteins be sufficiently mild to allow for the detection of the unaltered protein-bound pyrroles.

Figures 1 and 2 demonstrate that the administration of 2,5-HD to rats does result in the accumulation of pyrrolic substances in their body hairs. Figure 2 also demonstrates that these pyrrolic substances are associated with the dialyzed protein fraction of solubilized hair. These substances were present during the entire 45-d period of daily administration, and were still found to he present in hair samples 25 d after the cessation of 2,5-HD administration (Figure 1). These results indicate that this persistence or stability of these pyrroles should be adequate for purposes of any biological assay.

It should also he noted that this access to the pilary system of the 2,5-HD pyrrole precursor can take place via the hair bulbs, whose cells, during the period of hair growth (anagen phase), undergo proliferation and ultimately form the differentiated cells of the mature hair shaft (Montagna & Parakkal, 1974). This is shown in Figure 4, in representative photographs of our indirect immunofluorescence studies of frozen skin sections taken from the rats. These findings indicating the presence of protein-bound pyrrolic substances in the hair bulbs of treated rats are in accord with this concept.

Figure 3, demonstrating the ongoing pyrrole formation in the vibrissae of rats, indicates that the process of 2,5-HD uptake with subsequent formation of protein-bound pyrroles can occur during hair elongation (i.e., growth). The tissue response to DMAB (red color) does diminish in intensity, which demonstrates that some degree of alteration of the pyrrole adducts does take place with time, though at a slow rate. On the other hand, as long as there is a detectable positive pyrrole reaction at a location along the axis of a growing hair, the distance from the follicle can be utilized to determine the time in which the pyrrole was initially present at the point of origin. Figure

3, depicting the continual development of pyrrole substances with time along the length of the individual vibrissae, merely demonstrates the potential for monitoring exposure over extended periods. In practice, however, multiple hair samples must be assessed at any specific time point in order to accommodate the cyclic nature of the individual hair follicles (Montagna & Parakkal, 1974). At any one time, an individual hair fiber may be in the growing state (anaphase) or its final nongrowing club or catagen phase. Thus, the extent of the DMAB-positive portion of any one hair could be indicative of the actual time of exposure of the animals to chronic dosing or it could represent the time of onset of growth of that particular hair that occurred after the initiation of chronic exposure.

Our findings of pyrrolated hair proteins that respond to the DMAB reagent following their extraction (Figure 2) demonstrate the means by which such assessments could be made in humans (or other species) bearing pigmented hair. In these circumstances, a sample of hair sufficient to be representative of hairs in all phases of growth would be plucked at the skin surface and bundled to preserve the original tip–follicle orientation. Segments of the bundled hair of known length would be assayed for pyrrole-bound proteins following their solubilization. The segment most distal to the follicle that shows a positive DMAB reaction when factored by the known rate of hair growth should provide an estimate of the duration of exposure. It should be noted that the rate of hair growth is constant (Ebling et al., 1991). It has also been ascertained that when known amounts of 2,5-dimethylpyrrole were added to protein solutions of pigmented human hair, there was no interference with the colorimetric recovery by the pigment present (data not shown).

In contrast to the ongoing accumulation of pyrrole substance observed in the case of hair, the daily urinary excretion does not correlate with the number of the daily dosages. These two events, however, may ultimately be utilized in a cooperative manner. The presence of pyrrole-like substances in the urine of a subject could serve as a biomarker for an acute or recent exposure. This could then be followed with an examination of hair for an assessment of any chronic exposure.

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