0026-895X/86/050452-07$02.00/0

MOLECULAR PHARMACOLOGY, 30:452–458

Mechanisms of *In Vitro* Pyrrole Adduct Autoxidation in 2,5-Hexanedione-Treated Protein

ANTHONY P. DECAPRIO

Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201

Received November 18, 1985; Accepted August 14, 1986

SUMMARY

The neurotoxic γ-diketone, 2,5-hexanedione, reacts with axonal protein amine residues to form 2,5-dimethylpyrrole adducts. Current evidence implicates this reaction as the potentially critical step in γ-diketone neurotoxicity, although it is unclear whether pyrrole formation per se is sufficient to induce neuropathy or whether secondary autoxidative reactions are also required. The present *in vitro* study examines aspects of pyrrole formation and the secondary phenomena of chromophore development and covalent protein crosslinking in 2,5-hexanedione-treated protein. *p*-Dimethylaminobenzaldehyde (DMAB)-detectable pyrrole concentrations decreased linearly with time when pyrrolylated bovine serum albumin (pyrrole-BSA) was incubated under air, but remained unchanged following N_2 incubation. The air-induced decrease was accompanied by the appearance of chromophores and crosslinked protein. Covalent crosslinking of pyrrole-BSA was pH-dependent, with relatively increased intermolecular bridging at pH 7.4 as compared to pH 9.5. Chromophore formation and the loss in DMAB-detectable pyrrole were also accelerated at the lower pH. Autoxidative parameters were inhibited in the presence of a free radical scavenger (ascorbic acid) but induced by free radical initiators (potassium persulfate and 2,2′-azobis[2-amidinopropane hydrochloride]). *In vitro* incubation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of combinations of bovine serum albumin, ribonuclease, pyrrole-BSA, and pyrrolylated ribonuclease revealed that the intermolecular crosslinking pathway was mediated by pyrrole-pyrrole bridging. These findings demonstrate that the secondary autoxidative phenomena following pyrrole adduct formation in γ-diketone-treated protein proceed via pH-dependent, free radical-mediated mechanisms. If similar mechanisms are present *in vivo*, the results also suggest that intermolecular covalent crosslinking of pyrrolylated axonal protein may be less widespread and more specific than previously thought.

2,5-HD is the neurotoxic γ-diketone metabolite of the industrial solvents *n*-hexane and methyl *n*-butyl ketone (1, 2). This compound is thought to act directly upon the axon to induce a characteristic NF accumulation and, ultimately, nerve degeneration in certain long, large-diameter, myelinated nerve fibers. Previous studies have revealed the formation of 2,5-dimethylpyrrole adducts during the reaction of 2,5-HD with protein amine groups, both *in vitro* and *in vivo* (3–5). *In vitro* studies have also demonstrated the propensity of alkyl pyrrole adducts to undergo secondary autoxidative reactions, ultimately yielding covalently crosslinked, polymeric derivatives (3, 6).

Based upon the above findings, DeCaprio *et al.* (3, 4) suggested that pyrrolylation of the NF proteins would result in increases in hydrophobicity and tertiary structure alterations which could have a deleterious effect on axonal cytoskeletal function or transport. Graham *et al.* (6) alternatively proposed that the formation of autoxidative crosslinks between pyrrolylated NFs might result in similar effects. Evidence is available that the NF proteins undergo both pyrrolylation and subsequent autoxidative crosslinking following γ-diketone exposure (5, 7). Thus, it is currently unclear whether pyrrole adduct formation in axonal proteins per se is sufficient to induce neuropathy or whether secondary alterations of the pyrrole nucleus are also required.

As a first step toward selecting between these hypotheses, studies on the chemical and mechanistic aspects of pyrrole adduct autoxidation would be of value. In general, the oxidative chemistry of alkyl pyrroles is well documented (8–10), although specifically, the routes and products of the autoxidative reactions are obscure. These processes are thought to involve hydroperoxyl and alkoxyl free radical species which interact via addition or substitution mechanisms (8–10). Little information is available concerning the oxidation products of alkyl pyrroles in aqueous solution. Pyrrolizidine alkaloids are oxidized *in vivo* to electrophilic pyrrole derivatives which bind covalently with tissue components (11). Similar covalent binding was observed following *in vitro* microsomal activation of 1,3,4-trimethylpyrrole to an electrophilic (free radical?) species (12).

This work was supported by United States Public Health Service/National Institute of Occupational Safety and Health Research Grant OH-01972.

ABBREVIATIONS: 2,5-HD, 2,5-hexanedione; NF, neurofilament; BSA, bovine serum albumin; RN, ribonuclease; DMAB, *p*-dimethylaminobenzaldehyde; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ABAP, 2,2′-azobis(2-amidinopropane) hydrochloride.

Since the autoxidative reactions might be of importance in the ultimate mechanism of γ-diketone neuropathy, it was of interest to further explore details of this phenomenon. The present investigation employed an *in vitro* system using model proteins to facilitate examination of the mechanisms of complex pyrrole autoxidation reactions under simulated *in vivo* conditions. This report describes experimental conditions which were found to inhibit pyrrole adduct autoxidation *in vitro*. Evidence is also provided that chromophore development, intermolecular crosslinking, and possibly intramolecular crosslinking in pyrrolylated protein are pH-dependent, free radical-mediated processes.

Materials and Methods

Chemicals. 2,5-HD (Eastman Kodak Co., Rochester, NY) was >98% pure and was redistilled prior to use. RN (type A from bovine pancreas), potassium persulfate, $NaBH_4$, and ascorbic acid were obtained from Sigma Chemical Co., St. Louis, MO. Globulin-free BSA (Sigma Chemical Co.) was used for all experiments except the BSA-RN heteropolymer experiment, where BSA monomer (Miles Scientific, Naperville, IL) was employed. 2,5-Dimethylpyrrole and DMAB were obtained from Aldrich Chemical Co., Milwaukee, WI. ABAP was purchased from Polysciences, Inc., Warrington, PA.

Pyrrole assay. The colorimetric assay for 2,5-dimethylpyrrole adducts utilized a modification of the previously described DMAB method (3). Samples (0–100 μl) were diluted to 100 μl with distilled H_2O followed by the addition of 1.3 ml of 1% (w/v) SDS in H_2O and 100 μl of a reagent consisting of 1.5% (w/v) DMAB and 1% HCl (v/v) in methanol:H_2O (50:50). The absorbance was determined at 530 nm and standard curves were generated using 2,5-dimethylpyrrole. Pyrrole adduct levels were expressed as nmol of 2,5-dimethylpyrrole/mg of protein.

Inert atmosphere system. For *in vitro* incubations requiring the exclusion of air, a purging system consisting of a Cartesian manostat, high vacuum pump, Firestone valve (Aldrich Chemical Co.), and Teflon-sealed vacuum sample tubes (Pierce Chemical Co., Rockford, IL) was utilized with ultra-high purity N_2 as the inert gas. Samples were alternately exposed to vacuum (20 mm Hg) and N_2 for a total of six cycles, resulting in a theoretical final O_2 partial pressure of 5.55×10^{-5} mm Hg (13). Samples were repurged with N_2 after each opening.

Synthesis of pyrrole-BSA and pyrrole-RN. For experiments requiring pyrrolylated proteins, native proteins (10 mg/ml in 200 mM sodium phosphate buffer with 0.1% NaN_3, pH 7.4 or 9.5) were incubated with a 50-fold molar excess of 2,5-HD to protein lysine residues, based upon published amino acid composition data (14, 15). Incubation was performed under an inert atmosphere at 37° for 24–48 hr in the dark, followed by exhaustive dialysis (under N_2) versus 10 mM sodium phosphate buffer containing 0.05% NaN_3, pH 7.4 or 9.5. This procedure was determined to be effective in completely removing unreacted 2,5-HD. Pyrrolylated protein solutions were either used immediately or stored under N_2 at −80° for future use.

Effects of pH and incubation atmosphere on pyrrole autoxidation parameters. Relationships between chromophore development (A_{475nm}), intermolecular crosslinking, and incubation pH were examined using BSA treated with a 50:1 2,5-HD:lysine molar ratio for up to 67 hr under air. Following incubation, the A_{475nm} and pyrrole content of each sample were determined. In addition, samples of pyrrole-BSA (prepared as described above) in 200 mM sodium phosphate, 0.1% NaN_3 were incubated under air (37°) at either pH 7.4 or pH 9.5 for 40 hr, followed by SDS-PAGE (16). Intermolecular cross-

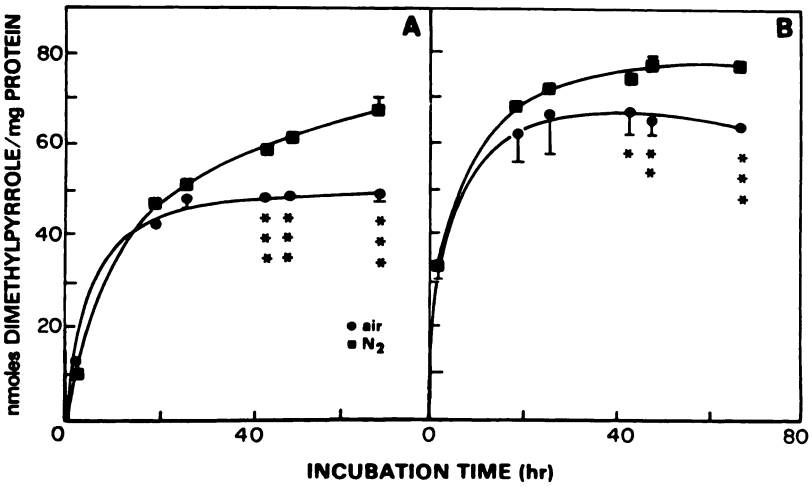

Fig. 1. DMAB-detectable pyrrole formation in BSA treated with 2,5-HD. BSA was incubated with 2,5-HD at pH 7.4 (A) or pH 9.5 (B) under air (●) or N_2 (■) as described under Materials and Methods. Each point represents the mean ± standard deviation of triplicate determinations from three experiments. Standard deviation lies within the *symbol* if not shown. *Asterisks* indicate a significant difference (Student's *t* test) between air and N_2 values, $p \leq 0.05$ (•), $p \leq 0.01$ (••), or $p \leq 0.001$ (•••).

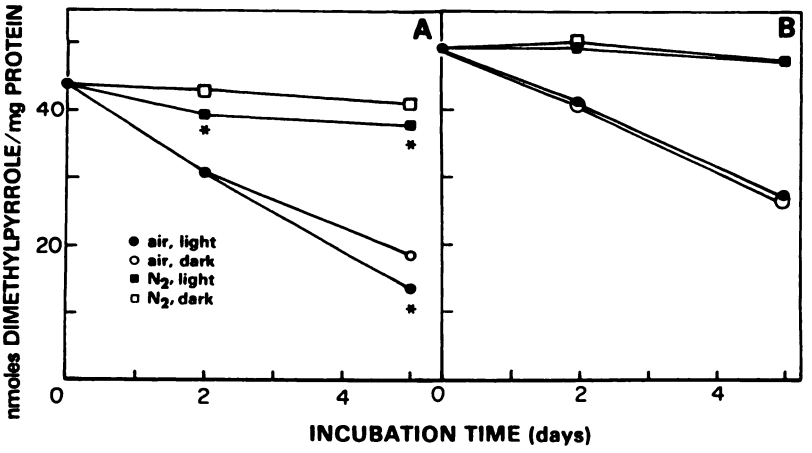

Fig. 2. DMAB-detectable pyrrole levels in pyrrole-BSA following incubation under combinations of air/N_2 and light/dark. Pyrrole-BSA was incubated at pH 7.4 (A) or pH 9.5 (B) as described under Materials and Methods. Each point represents the mean of duplicate determinations from three separate experiments. Standard deviation lies within the symbol for all points. •, significant difference (Student's *t* test) between values for light versus dark incubation, $p \leq 0.05$.

linking in these samples was quantitated by densitometric scanning of gels stained with Fast Green FCF (17). Densitometry was performed using a Beckman CDS-200 scanning densitometer at a wavelength of 600 nm. Intermolecular crosslinking was expressed as the percentage of total protein which was present as monomer, dimer plus trimer, and higher polymer. The control BSA used in this experiment normally contained a small fraction (~12%) of higher polymer. In a related experiment (pH reversal experiment), samples of pyrrole-BSA, previously synthesized at either pH 7.4 or pH 9.5, were incubated (200 mM sodium phosphate buffer containing 0.1% NaN_3, 37°) under air at pH 9.5 or 7.4, respectively, for up to 48 hr. SDS-PAGE was then performed in order to assess intermolecular crosslinking as described above.

In order to determine the effects of air and light on DMAB-detectable pyrrole levels, samples of pyrrole-BSA were incubated under combinations of air/N_2 and light/dark for up to 5 days at pH 7.4 or 9.5. Light incubation was performed using fluorescent illumination. Pyrrole adduct levels were measured in each sample using the DMAB assay and plotted as a function of time.

Effects of ascorbic acid and radical initiators on pyrrole autoxidation. The influence of the antioxidant ascorbic acid and the free radical initiators potassium persulfate and ABAP on pyrrole autoxidation was determined. Aliquots of freshly prepared (100 mM or 1 M in H_2O) ascorbic acid were added to aliquots of control or pyrrole-BSA in 200 mM sodium phosphate buffer containing 0.1% NaN_3, pH 7.4 or 9.5, to yield final concentrations of 1, 10, or 100 mM. Samples were incubated at 37° under air for 24 hr, followed by assessment of DMAB-detectable pyrrole, A_{475nm}, and covalent crosslinking by SDS-PAGE. Similarly, aliquots of freshly prepared (100 mM in H_2O) potassium persulfate or ABAP were added to control or pyrrole-BSA in buffer to final concentrations of 1 or 5 mM, respectively. Incubation was performed at 37° in the dark under N_2 for 24 hr, followed by SDS-PAGE, pyrrole assay, and measurement of A_{475nm} as described above.

Determination of residues involved in intermolecular crosslinking. In order to gain insight into the nature of the intermolecular crosslinking process, various combinations of BSA, pyrrole-BSA (synthesized from BSA monomer), RN, and pyrrole-RN were incubated under air (37°, 24 hr, pH 7.4). Protein:protein molar ratios in the incubation mixtures were varied over a 5-fold range. Proteins were then separated by SDS-PAGE and stained with Coomassie blue R-250 in order to observe the appearance of protein heteropolymers from incubated mixtures of pyrrolylated and/or control species.

Results

Effects of incubation atmosphere on DMAB-detectable pyrrole. The time courses of DMAB-detectable pyrrole formation in BSA incubated with 2,5-HD (50:1 2,5-HD:lysine molar ratio) at pH 7.4 or 9.5 are illustrated in Fig. 1. Absolute pyrrole concentrations were higher at pH 9.5 than at pH 7.4 at all times examined. The initial rate of pyrrole formation was also dependent upon the incubation pH, with higher rates observed at pH 9.5. Incubation under N_2 resulted in significantly greater DMAB-detectable pyrrole concentrations at either pH after 48 hr, relative to those generated under air. In contrast, the initial rate of pyrrole formation at a particular pH was essentially unaffected by N_2 incubation.

Fig. 2 illustrates the effects of air/N_2 and light/dark incubation on DMAB-detectable pyrrole concentrations in pyrrole-BSA at pH 7.4 and 9.5. With air/dark incubation, a progressive decline in detectable pyrrole was apparent at both pH values during the incubation period. This decline was greater at pH 7.4 (68%) than at pH 9.5 (43%). Fluorescent illumination slightly accelerated this decrease at pH 7.4 but not at pH 9.5. In contrast, incubation under N_2/dark conditions resulted in no significant loss in pyrrole adduct levels at either pH. The decline in DMAB-detectable pyrrole was accompanied by the

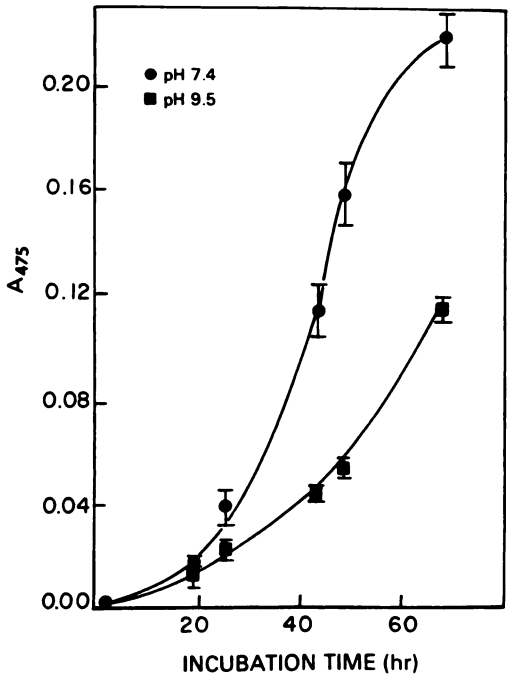

Fig. 3. Chromophore appearance (A_{475nm}) in mixtures of BSA and 2,5-HD. BSA was incubated at pH 7.4 (●) or pH 9.5 (■) under air as described under Materials and Methods. Each point represents the mean ± standard deviation from three separate experiments. Standard deviation lies within the *symbol* if not shown.

TABLE 1

Effects of incubation pH on intermolecular autoxidative crosslinking of pyrrole-BSA[a]

Incubation pH	Sample	Crosslinking[b]		
		Monomer	Dimer + Trimer	Polymer
7.4	Control BSA[c]	88.3 ± 0.6	9.3 ± 0.9	2.4 ± 1.0
	Pyrrole-BSA	53.0 ± 3.6	23.3 ± 2.0	23.3 ± 3.3
9.5	Control BSA	87.7 ± 0.8	9.5 ± 0.9	2.7 ± 0.7
	Pyrrole-BSA	69.6 ± 2.7[d]	21.9 ± 2.8	7.2 ± 0.7[d]

[a] BSA (5 mg/ml in 200 mM sodium phosphate with 0.1% NaN_3, pH 7.4) was incubated with 2,5-HD for 24 hr followed by exhaustive dialysis under N_2 (see Materials and Methods). The resulting pyrrole-BSA was reincubated under air for 40 hr at either pH 7.4 or pH 9.5, followed by SDS-PAGE.

[b] Determined by densitometric quantitation of Fast Green FCF-stained protein bands in SDS-PAGE gels. Values are expressed as the percentage of total protein present as monomer, dimer + trimer, and higher polymer, respectively (see Fig. 5). Data represent the mean ± standard deviation of duplicate determinations from three experiments.

[c] Control BSA was incubated under the same conditions employed for pyrrole-BSA, but without 2,5-HD.

[d] Significantly different (Student's *t* test) from pyrrole-BSA value at pH 7.4, $p \leq 0.01$. All pyrrole-BSA values were significantly different from corresponding control BSA values at $p \leq 0.001$.

appearance of an orange chromophore (λ_{max} = 475 nm) in the incubation mixtures. Fig. 2 also indicates that fluorescent illumination resulted in a small but significant decrease in detectable pyrrole levels under N_2 incubation at pH 7.4 but not at pH 9.5 after 5 days.

Quantitation of chromophore development and crosslinking in pyrrole-BSA. Previous studies (3, 6) had revealed that several phenomena accompany pyrrole formation in protein treated with 2,5-HD under air, i.e., chromophore appearance (A_{475nm}), intramolecular crosslinking, and intermolecular crosslinking. Results of quantitative studies of these phenomena as a function of incubation time at pH 7.4 and 9.5 are

shown in Fig. 3 and in Table 1. The appearance of color was enhanced at pH 7.4 compared to pH 9.5 (Fig. 3), despite the increased pyrrole adduct concentration at the higher pH. Both curves exhibited an early lag period of approximately 10 hr during which color formation was minimal, followed by an accelerated rate of color development.

The relative extent of intermolecular covalent crosslinking was highly dependent upon the incubation pH (Table 1). Conversion of pyrrole-BSA into dimers, trimers, and higher polymers was apparent at pH 7.4, whereas significantly lower levels of intermolecular crosslinking were seen at pH 9.5 (Table 1). Densitometric quantitation revealed that, although intermolecular crosslinking did occur at pH 9.5, it was restricted primarily to the formation of BSA dimers and trimers. In contrast, incubation at pH 7.4 produced these species in addition to very high molecular weight BSA polymers. An increased migration rate and some spreading of the band corresponding to the BSA monomer were also noted in pyrrole-BSA incubated at pH 9.5 as compared to control BSA (see Fig. 4b). This effect has previously been attributed to intramolecular crosslinking, a process which would result in a lower effective hydrodynamic volume and enhanced mobility of protein monomers in SDS-PAGE (18–20). In contrast, such increased migration was not prominent in samples incubated at pH 7.4 (see Fig. 4a). Treatment with $NaBH_4$ resulted in a loss of color but no change in the level of protein crosslinking (data not shown). In the pH reversal experiment, substantial intermolecular crosslinking was observed when pyrrole-BSA synthesized (under N_2) at pH 9.5 was incubated (under air) at pH 7.4. In contrast, pyrrole-BSA initially synthesized at pH 7.4 exhibited little intermolecular crosslinking when incubated at pH 9.5. These findings indicate that the extent of induced crosslinking was dependent upon the incubation (autoxidation) pH rather than that at which the pyrrole-BSA was initially synthesized.

Effects of ascorbic acid and radical initiators. The effects of ascorbic acid treatment on covalent crosslinking of pyrrole-BSA are illustrated in Fig. 4. The presence of 10 or 100 mM ascorbate substantially inhibited intermolecular crosslinking (Fig. 4a), as judged by the relative decrease in the level of polymeric protein above the BSA monomer band. Concentrations of 10 and 100 mM ascorbate also decreased the migration rate of the pyrrole-BSA monomer band, suggesting a possible reduction of intramolecular crosslinking (Fig. 4b). The decreases in crosslinking were accompanied by relative increases in DMAB-detectable pyrrole concentrations and decreases in A_{475nm} (not shown).

Additional evidence for a mechanism of free radical-mediated crosslinking was obtained using potassium persulfate as a source of radical initiating species. Fig. 5 demonstrates the effect of 1 mM persulfate on incubation mixtures of control BSA or pyrrole-BSA at pH 7.4 under N_2. Substantial intermolecularly crosslinked polymeric pyrrole-BSA was induced by persulfate in the absence of air (Fig. 5, *lane 3*), whereas only minor dimerization and trimerization was seen without the radical initiator (Fig. 5, *lane 2*). The two bands appearing in the region of the gel between dimer and high polymer most likely represent linear and cyclic forms of the pyrrole-BSA trimer (Fig. 5, *lane 2*). Treatment of control BSA with persulfate at pH 7.4 was without effect (Fig. 5, *lane 1*). Similar, although less marked, effects were observed following ABAP treatment (not shown).

Table 2 illustrates the effects of persulfate and ABAP on DMAB-detectable pyrrole and A_{475nm}. Incubation of pyrrole-BSA with these radical initiators under N_2 at pH 7.4 resulted in the appearance of an orange color with the same λ_{max} (475 nm) as that observed following air incubation. This color could be essentially eliminated by the addition of $NaBH_4$ (data not shown). DMAB-detectable pyrrole levels were also significantly

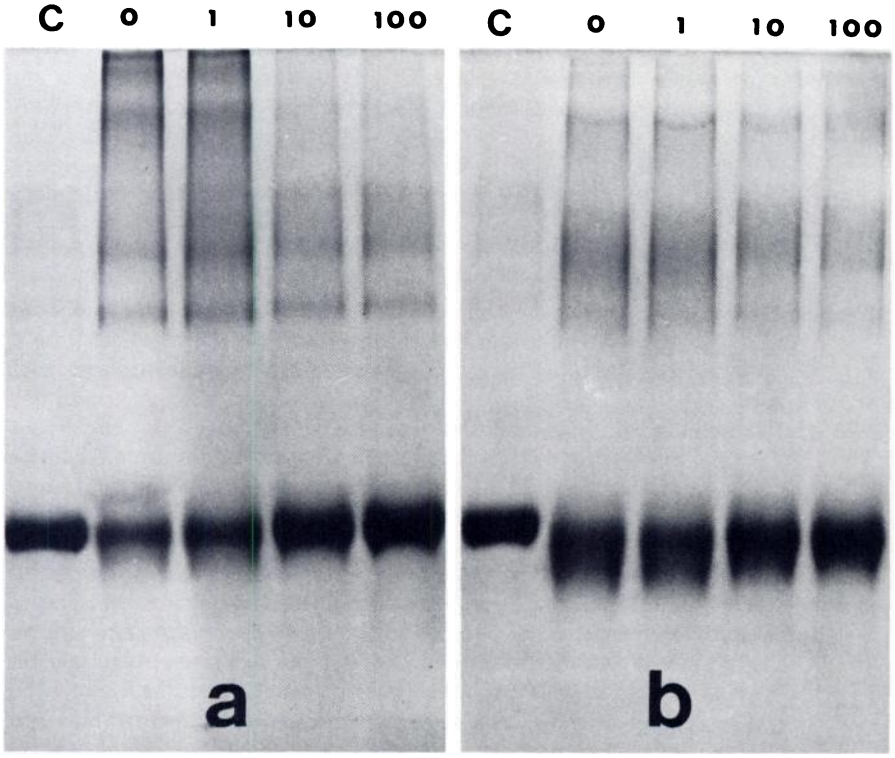

Fig. 4. Effect of ascorbic acid on covalent crosslinking of pyrrole-BSA. Control BSA and pyrrole-BSA were incubated under air at either pH 7.4 (gel a) or pH 9.5 (gel b) as described under Materials and Methods, followed by SDS-PAGE and Coomassie blue R-250 staining. Control BSA (lane 1), pyrrole-BSA incubated with 0, 1, 10, or 100 mM ascorbic acid final concentration (lanes 2–5). Intermolecular crosslinking can be assessed by the increase in polymeric material above the BSA monomer band. A 7.5% acrylamide separating gel with a 5% stacking gel was used.

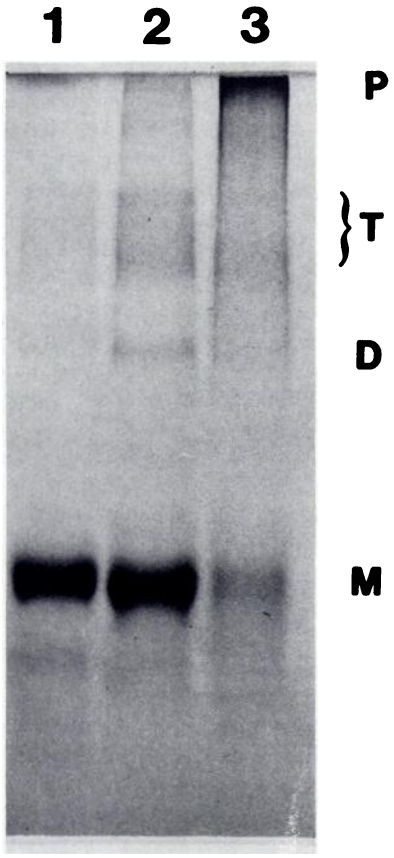

Fig. 5. Effect of persulfate on intermolecular crosslinking of pyrrole-BSA. Control BSA and pyrrole-BSA were incubated under N_2 with or without added potassium persulfate (1 mM) as described under Materials and Methods, followed by SDS-PAGE. (*Lane 1*, control BSA with persulfate, pH 7.4. *Lane 2*, pyrrole-BSA, no persulfate, pH 7.4. *Lane 3*, pyrrole-BSA with persulfate, pH 7.4. Substantial induction of high molecular weight polymer can be seen in pyrrole-BSA samples treated with persulfate (*lane 3*). In contrast, only minor dimer and trimer formation is seen without persulfate (*lane 2*). Control BSA is unaffected by the persulfate treatment (*lane 1*). Protein bands are identified at *right* as follows: *P*, high molecular weight BSA polymer; *T*, BSA trimer; *D*, BSA dimer; *M*, BSA monomer. Gel conditions are the same as in Fig. 4.

TABLE 2

Effect of radical initiators on pyrrole-BSA autoxidation

Incubation pH[a]	Initiator[b]	DMAB-detectable pyrrole[c]	A_{475nm}[d]
		nmol pyrrole/mg protein	
7.4	None	52.5 ± 7.8	0.042 ± 0.010
	Persulfate	21.5 ± 0.1[e]	1.153 ± 0.149[f]
	ABAP	40.1 ± 3.0[g]	0.255 ± 0.039[f]
9.5	None	57.9 ± 4.3	0.025 ± 0.006
	Persulfate	43.1 ± 1.1[e]	0.248 ± 0.004[f]
	ABAP	58.2 ± 5.6	0.058 ± 0.033

[a] Pyrrole-BSA (5 mg/ml in 200 mM sodium phosphate buffer with 0.1% NaN_3) was incubated under N_2 (37°) for 24 hr.

[b] Aliquots of freshly prepared solutions of initiator in H_2O were added to give a final 1 mM (persulfate) or 5 mM (ABAP) concentration.

[c] Data represent means ± standard deviations from two to four separate experiments.

[d] Values are means ± standard deviations from two to four experiments.

[e] Significantly different (Student's *t* test) from sample with no initiator, $p \leq 0.01$.

[f] $p \leq 0.001$.

[g] $p \leq 0.05$.

reduced following persulfate and ABAP treatment at pH 7.4. In contrast, only persulfate was effective in producing these changes at pH 9.5.

Identification of residues involved in intermolecular crosslinking. In order to identify the amino acid moieties involved in the intermolecular crosslinking process, various incubation mixtures of BSA, pyrrole-BSA, RN, and pyrrole-RN were analyzed by SDS-PAGE to detect the presence of protein heteropolymers. Fig. 6 presents the results of this study. Incubation of control RN with pyrrole-BSA (Fig. 6, *lane 4*) or control BSA with pyrrole-RN (Fig. 6, *lane 6*) at equimolar ratios did not result in the observable formation of protein heteropolymers. This was true even when 5:1 molar ratios of control:pyrrolylated protein (Fig. 6, *lanes 5* and *7*) were employed in an attempt to increase the likelihood of crosslinking. Only in the case of the pyrrole-BSA + pyrrole-RN mixtures were heteropolymers observed (Fig. 6, *lane 10*). No crosslinking was observed in samples prior to incubation under air (not shown).

In addition to pyrrole-RN and pyrrole-BSA monomer bands and pyrrole-RN polymer bands (RN_{2-4}), heteropolymers apparently corresponding to pyrrole-BSA bound to one (BSA:RN), to two (BSA:RN_2), and to three pyrrole-RN molecules (BSA:RN_3) were clearly seen. Although not specifically indicated in Fig. 6, bands corresponding to pyrrole-BSA homopolymers (BSA_{2-4}) were visible (Fig. 6, *lane 8*), along with higher pyrrole-RN polymers (RN_{5-7}) (Fig. 6, *lane 9*). These findings indicate that, in this *in vitro* system, intermolecular crosslinking in pyrrolylated protein probably occurs via pyrrole-pyrrole bridging.

Discussion

The formation of alkyl pyrrole adducts from the reaction of γ-diketones with protein amine groups is well documented in a number of *in vitro* and *in vivo* systems (3–5). Secondary autoxidative modification of pyrrole adducts has also been described (3, 6), although many aspects of this phenomenon remain obscure. Such secondary reactions are manifested by the appearance of chromophores and by intermolecular and intramolecular protein crosslinking. In view of the potential significance of these secondary reactions to the molecular mechanism of γ-diketone neuropathy, it was of interest to explore further their characteristics.

Results from the present study support a free radical-induced mechanism for the secondary observations of chromophore development and covalent crosslinking in pyrrolylated protein following *in vitro* exposure to air. A third, previously unreported phenomenon, i.e., a decrease in DMAB-detectable pyrrole concentrations, appears to be similarly mediated. This observation most likely reflects both a radical-mediated deactivation of the pyrrole ring toward electrophilic attack by DMAB and a loss of accessibility of certain adducts to DMAB due to tertiary structure changes in the protein and/or intramolecular crosslinking. The ability of fluorescent illumination to either induce (under N_2 atmosphere) or accelerate (under air atmosphere) the decrease in DMAB-detectable pyrrole is consistent with an increased rate of initiator formation (21). Incubation under N_2 or in the presence of the antioxidant and free radical scavenger ascorbic acid (22) was found to significantly reduce these autoxidative changes. Complete suppression of pyrrole autoxida-

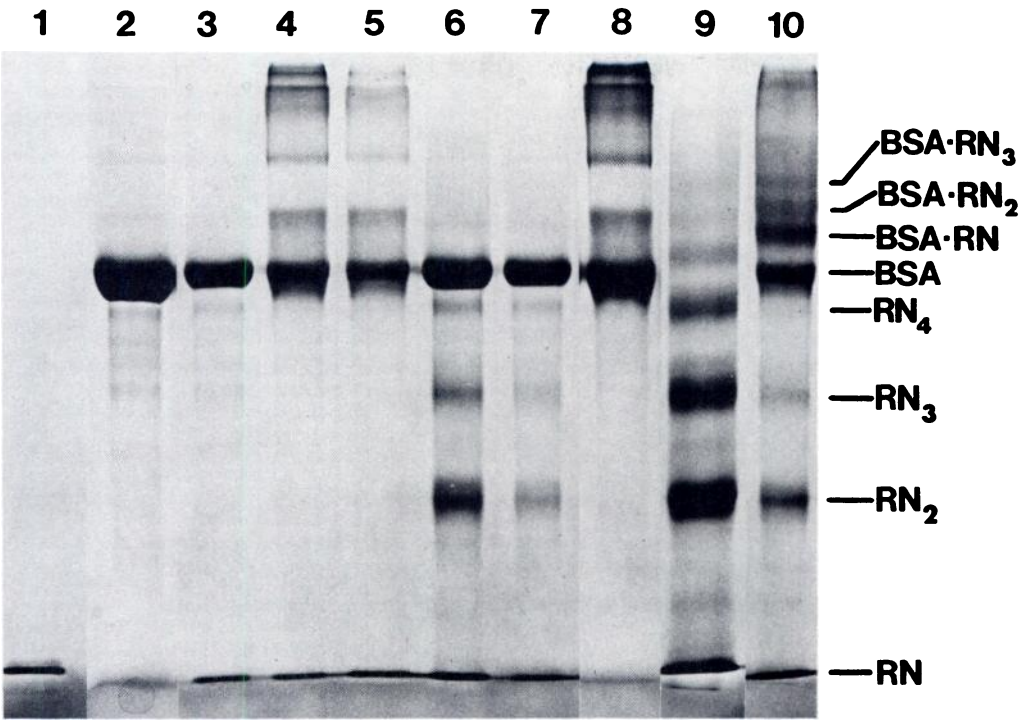

Fig. 6. Formation of pyrrole-pyrrole crosslinks during incubation of pyrrole-BSA with pyrrole-RN. Combinations of BSA, RN, pyrrole-BSA, and pyrrole-RN were incubated under air as described under Materials and Methods, followed by SDS-PAGE. Samples were as follows: RN (*lane 1*), BSA (*lane 2*), BSA + RN, equimolar mixture (*lane 3*); pyrrole-BSA + RN, 1:1 and 1:5 molar ratios (*lanes 4* and *5*); pyrrole-RN ± BSA, 1:1 and 1:5 molar ratios (*lanes 6* and *7*); pyrrole-BSA (*lane 8*); pyrrole-RN (*lane 9*); and pyrrole-BSA + pyrrole-RN, equimolar mixture (*lane 10*). Protein bands are identified (based on molecular weights) at *right* as follows: *BSA*, pyrrole-BSA monomer; *RN*, RN_{2-4} pyrrole-RN monomer, dimer, trimer, and tetramer; ($BSA \cdot RN$, $BSA \cdot RN_{2,3}$) pyrrole-BSA: pyrrole-RN heteropolymers. Note that only with pyrrole-RN + pyrrole-BSA mixtures (*lane 10*) are protein heteropolymers observed. Bands corresponding to homopolymers BSA_{2-4} and RN_{5-7} can also be seen in *lanes 8* and *9*. A 10% acrylamide gel with 5% stacking gel was used.

tion in this system thus appears to require a potent antioxidant in addition to rigid exclusion of O_2.

Studies utilizing the radical initiators potassium persulfate and ABAP provide further evidence for the involvement of free radicals in pyrrole adduct autoxidation. The addition of these compounds to incubation mixtures of pyrrole-BSA under N_2 resulted in rapid induction of effects similar to those observed following prolonged air incubation without added initiators. In aqueous solution, these compounds respectively yield persulfate radical anions and carbon- or nitrogen-centered radical cations (23, 24), which can apparently substitute for O_2-derived species as initiators of pyrrole autoxidation. The finding that structurally diverse initiators have similar effects in this system suggests that the chemical nature of the initiating species may not be critical. Thus, pyrrole adduct autoxidation *in vivo* may occur as a result of reaction with radicals present in living systems (25).

The detailed chemistry underlying these *in vitro* autoxidative reactions remains speculative. Evidence from the present and previous investigations suggests an initial lag phase which is accelerated in the presence of O_2 (3, 26, 27). This early phase is presumably required for the formation of initiators of unknown structure (28, 29), which may then attack unoxidized pyrrole moieties by either abstraction of ring or side chain hydrogen atoms or by addition at ring carbons. Both processes have been reported using various model pyrrole systems (30–32), and multiple radical products would therefore be expected. The resultant pyrrole radicals could then attack O_2 or other pyrrole moieties in a series of propagation steps. Termination reactions would ultimately yield pyrrole hydroperoxides and other complex oxidized and polymeric derivatives. The direct reaction of O_2 with the pyrrole ring to yield diradical species (33) probably does not occur under the conditions used in the present study, since this process requires photoactivating agents.

Some conclusions can be made concerning the basis for chromophore development and the precise structural nature of the intermolecular crosslink from the present data. The increase in A_{475nm} observed with incubation of pyrrole-BSA at pH 7.4 correlated with the level of intermolecular crosslinking determined by SDS-PAGE. This finding suggests that the observed color could be an inherent property of the crosslink, as might be expected with a dipyrylmethene structure (λ_{max} = 450–500 nm) (9, 10). Although well known in pyrrole chemistry, dipyrrylmethenes have not been demonstrated as products of pyrrole autoxidation or other free radical reactions. Alternatively, color might be the result of the formation of pyrrolone derivatives from the breakdown of pyrrole hydroperoxides. Crosslinking could then be attributed to the formation of bipyrrolones analogous to those described from the autoxidation of 2,3,4-trimethylpyrrole (34). The loss of color upon treatment with $NaBH_4$ would be consistent with this option, since keto functions are readily reduced under these conditions, whereas the carbon-carbon double bonds of dipyrylmethenes are not (35). However, $NaBH_4$ also eliminated color in pyrrole-BSA solutions treated with persulfate under N_2. Under conditions of O_2 exclusion, pyrrolones would not be expected products. It is apparent that additional research is required to unequivocally establish structural details concerning the products of these autoxidative reactions.

A marked pH dependence was observed for chromophore formation and the loss of DMAB-detectable pyrrole in pyrrole-BSA under air. Both of these processes were enhanced at pH 7.4 as compared to pH 9.5. In addition, added free radical initiators were more effective in inducing these changes at the lower pH. The mechanisms behind this pH dependence are unclear, although increasingly acidic conditions are known to promote pyrrole oxidation in organic systems (8, 9). Alternatively, the initiating species may be more stable at the lower

pH, although no specific evidence is available to support this. The relative increase in intermolecular crosslinking at pH 7.4 is more easily explained, since it most likely reflects a decreased repulsion between the acidic (pI < 5) BSA molecules as a function of decreasing pH. Whether or not the absolute number of crosslinks (intermolecular plus intramolecular) was increased at the lower pH could not be determined from the present data. Such a finding would be consistent with a general acceleration of pyrrole autoxidation with decreasing pH. Some evidence for a possible enhancement of intramolecular crosslinking at pH 9.5 was obtained in the present study, in the form of increased migration rates of pyrrole-BSA monomer in SDS-PAGE (18–20). If these findings are confirmed using more sensitive analytical techniques (e.g., sedimentation velocity, circular dichroism), then variation of pH in this *in vitro* system may provide a convenient method for separately exploring the two modes of autoxidative crosslinking.

The present *in vitro* investigation has provided information on the pyrrole autoxidation phenomenon and its possible mechanism, although additional research is required to extend these findings to physiological systems following γ-diketone exposure. Perhaps the most significant finding is that intermolecular crosslinking appears to be mediated by pyrrole-pyrrole bridging. Earlier speculation had suggested the participation of protein sites other than the pyrrole adduct in the crosslinking process (6). If similar mechanisms are present *in vivo*, this finding substantially limits the number of protein sites capable of covalent interaction and argues against a widespread, nonspecific crosslinking of NF proteins following γ-diketone exposure. Although it is not yet possible to determine whether pyrrole formation in axonal proteins is sufficient to cause neuropathy or whether secondary autoxidative reactions are also required, additional clues to this question have been provided. Data from the present study should aid in the design of novel γ-diketone derivatives which would form pyrroles with decreased susceptibility to secondary autoxidation. Such derivatives offer the greatest potential for ultimate resolution of this question.

References

1. Spencer, P. S., and H. H. Schaumburg. Experimental neuropathy produced by 2,5-hexanedione—a major metabolite of the neurotoxic industrial solvent methyl *n*-butyl ketone. *J. Neurol. Neurosurg. Psychiatry* **38**:771–775 (1975).
2. Couri, D., and M. Milks. Toxicity and metabolism of the neurotoxic hexacarbons *n*-hexane, 2-hexanone, and 2,5-hexanedione. *Annu. Rev. Pharmacol. Toxicol.* **22**:145–166 (1982).
3. DeCaprio, A. P., E. J. Olajos, and P. Weber. Covalent binding of a neurotoxic *n*-hexane metabolite: conversion of primary amines to substituted pyrrole adducts by 2,5-hexanedione. *Toxicol. Appl. Pharmacol.* **65**:440–450 (1982).
4. DeCaprio, A. P., N. L. Strominger, and P. Weber. Neurotoxicity and protein binding of 2,5-hexanedione in the hen. *Toxicol. Appl. Pharmacol.* **68**:297–307 (1983).
5. DeCaprio, A. P., and E. A. O'Neill. Alterations in rat axonal cytoskeletal proteins induced by *in vitro* and *in vivo* 2,5-hexanedione exposure. *Toxicol. Appl. Pharmacol.* **78**:235–247 (1985).
6. Graham, D. G., D. C. Anthony, K. Boekelheide, N. A. Maschmann, R. G. Richards, J. W. Wolfram, and B. R. Shaw. Studies of the molecular pathogenesis of hexane neuropathy. II. Evidence that pyrrole derivatization of lysyl residues leads to protein crosslinking. *Toxicol. Appl. Pharmacol.* **64**:415–422 (1982).
7. Lapadula, D. M., R. D. Irwin, E. Suwita, and M. B. Abou-Donia. Crosslinking of neurofilament proteins of rat spinal cord *in vivo* after administration of 2,5-hexanedione. *J. Neurochem.* **46**:1843–1850 (1986).
8. Gardini, G. P. The oxidation of monocyclic pyrroles, in *Advances in Heterocyclic Chemistry* (A. R. Katritzsky and A. J. Boulton, eds.), Vol. 15. Academic Press, New York, 67–98 (1973).
9. Jones, R. A., and G. P. Bean. *The Chemistry of Pyrroles.* Academic Press, New York (1977).
10. Jackson, A. H. Pyrroles, in *Comprehensive Organic Chemistry* (J. F. Stoddart, ed.), Vol. 1. Pergamon Press, New York, 275–320 (1979).
11. Mattocks, A. R. Toxicity of pyrrolizidine alkaloids. *Nature (Lond.)* **217**:723–728 (1968).
12. Guengerich, F. P., and M. B. Mitchell. Metabolic activation of model pyrroles by cytochrome P-450. *Drug Metab. Dispos.* **8**:34–38 (1980).
13. Shriver, D. F. *The Manipulation of Air-Sensitive Compounds.* McGraw-Hill, New York, 141–163 (1969).
14. Peters, T. Serum albumin: recent progress in the understanding of its structure and biosynthesis. *Clin. Chem.* **23**:5–12 (1977).
15. Mitra, S., and R. G. Lawton. Reagents for the crosslinking of proteins by equilibrium transfer alkylation. *J. Am. Chem. Soc.* **101**:3097–3110 (1979).
16. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
17. Gorovsky, M. A., K. Carlson, and J. L. Rosenbaum. Simple method for quantitative densitometry of polyacrylamide gels using Fast Green. *Anal. Biochem.* **35**:359–370 (1970).
18. Dunker, A. K., and A. J. Kenyon. Mobility of sodium dodecyl sulphate-protein complexes. *Biochem. J.* **153**:191–197 (1975).
19. Huang, C., and F. M. Richards. Reaction of a lipid-soluble, unsymmetrical, cleavable, cross-linking reagent with muscle aldolase and erythrocyte membrane proteins. *J. Biol. Chem.* **252**:5514–5521 (1977).
20. Ji, T. H. The application of chemical crosslinking for studies on cell membranes and the identification of surface reporters. *Biochem. Biophys. Acta* **559**:39–69 (1979).
21. Nonhebel, D. C., and J. C. Walton. *Free-Radical Chemistry.* Cambridge, University Press, London, 10–28 (1974).
22. Halliwell, B., and J. M. C. Gutteridge. Oxygen radicals and the nervous system. *Trends Neurol. Sci.* **8**:22–26 (1985).
23. Dagliotti, L., and E. Hayon. Flash photolysis of persulfate in aqueous solutions. Study of the sulfate and ozonide radical anions. *J. Phys. Chem.* **71**:2511–2516 (1967).
24. Dougherty, T. J. Chemistry of 2,2′-azobisisobutyramidine hydrochloride in aqueous solution: a water-soluble azo initiator. *J. Am. Chem. Soc.* **83**:4849–4853 (1961).
25. Freeman, B. A., and J. D. Crapo. Free radicals and tissue injury. *Lab. Invest.* **47**:412–426 (1982).
26. Oswald, A. A., and F. Noel. Role of pyrroles in fuel instability. *J. Chem. Eng. Data.* **6**:294–301 (1961).
27. Smith, E. B., and H. B. Jensen. Autoxidation of three 1-alkylpyrroles. *J. Org. Chem.* **32**:3330–3334 (1967).
28. Walling, C. *Free Radicals in Solution.* John Wiley and Sons, New York, 397–466 (1957).
29. Lloyd, W. G. Autoxidations, in *Methods in Free Radical Chemistry* (E. S. Huyser, ed.), Vol. 4. Marcel Dekker, New York, 1–131 (1973).
30. Conant, J. B., and B. F. Chow. The addition of free radicals to certain dienes, pyrrole, and maleic anhydride. *J. Am. Chem. Soc.* **55**:3475–3479 (1933).
31. Bridger, R. F., and G. A. Russell. Directive effects in the attack of phenyl radicals on carbon-hydrogen bonds. *J. Am. Chem. Soc.* **85**:3754–3765 (1963).
32. Gritter, R. J., and R. J. Chriss. Free-radical reaction of pyrroles. *J. Org. Chem.* **29**:1163–1167 (1964).
33. Quistad, G. B., and D. A. Lightner. On the photo-oxidation of 3,4-diethyl-2-methylpyrrole. *Tetrahedron Lett.* **46**:4417–4420 (1971).
34. Höft, E., A. R. Katritzsky, and M. R. Nesbit. The autoxidation of alkylpyrroles. *Tetrahedron Lett.* **32**:3041–3044 (1967).
35. Brown, H. C. *Hydroboration.* W. A. Benjamin, Inc., New York, 242–247 (1963).

Send reprint requests to: Dr. A. P. DeCaprio, Laboratory of Biochemical and Genetic Toxicology, Wadsworth Center for Laboratories and Research, New York State Department of Health, Empire State Plaza, Albany, NY 12201.