Arch. Environ. Contam. Toxicol. 17, 537–542 (1988)

Archives of Environmental Contamination and Toxicology

In Vitro Inactivation of Plasma α_1-Proteinase Inhibitor by Epoxides and 1,2-Dihaloethanes

G. A. S. Ansari, Jose C. Gan, and Brian K. Barton

Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, Texas 77550

Abstract. The inactivating effects of three epoxides and two dihaloethanes on the proteinase inhibitory activities of α_1-proteinase inhibitor, as well as those of the plasma itself, were investigated. All of the three epoxides (styrene oxide, ethylene oxide and propylene oxide), individually, inactivated the elastase inhibitory or trypsin inhibitory activity of proteinase inhibitor, and that of whole plasma. Styrene oxide is the most effective inactivator of either purified α_1-proteinase inhibitor or plasma, followed by ethylene oxide and propylene oxide in a decreasing order of potency. The dihaloethanes, 1,2-dichloroethane and 1,2-dibromoethane, also inactivated the proteinase inhibitory activities of α_1-proteinase inhibitor with the former being somewhat more effective than the latter. Concomitant modification of the amino group was also observed with these chemicals.

Occupational exposure to chemicals results in various diseases (Parmeggiani 1983; Kirsch-Volders 1984). Although attempts have been made to reduce the chemical exposure in a working environment, it has not been completely eliminated. Medical surveillance by physical health and haematological examinations are used to monitor the working population who are exposed to chemicals. However, by the time the toxic manifestations are detected by these means, extensive damage may have already occurred, and thus be too late for reversal or prevention. Biological monitoring of chemical exposure preferably addresses this problem for the occupationally exposed population. Procedures should be developed which can eventually be used for biological monitoring of occupationally exposed populations by analyzing changes in plasma proteins.

There are several advantages in using plasma proteins as a marker of chemical exposure. Namely, (a) plasma proteins will come in contact with chemicals and/or their metabolites as soon as they enter the circulation irrespective of the route of administration; (b) plasma proteins serve as carriers for the transport of hydrophobic compounds; (c) plasma can be easily obtained from exposed individuals.

A few preliminary studies have recently appeared in this direction. Marshall and Vesterberg (1983) have analyzed serum protein patterns by two-dimensional gel electrophoresis after exposing rats to trichloromethylene, dimethylformamide, and carbon tetrachloride, and found that a polypeptide (MW 45,000, pI 4.3) increased in concentration and was tentatively identified as α_1-acid glycoprotein. This is not surprising, since α_1-acid glycoprotein belongs to a family of acute phase proteins and the concentrations of these proteins increase in response to inflammation or stress (Piafsky 1980). Hemminki (1986) observed covalent binding of styrene oxide to human serum proteins and haemoglobin and attributed the binding to the cysteine after identifying the cysteine adducts of styrene from protein hydrolysates. Earlier studies from this laboratory have shown that α_1-PI is inactivated by the aldehydes found in cigarette smoke (Gan and Ansari 1986, 1987). We have extended these studies to include chemicals which are extensively used in the industry (Reisch 1987). In the present commu-

Address correspondence to G. A. S. Ansari

nication, we report *in vitro*, the reduction of proteinase inhibitory activity of α_1-proteinase inhibitor (α_1-PI) and unfractionated plasma by epoxides and 1,2-dihaloethanes. The inactivation occurs concomitant to the modification or disappearance of free amino groups in the proteins.

Materials and Methods

Pancreatic elastase, trypsin, human plasma α_1-proteinase inhibitor, *N*-succinyl-$(Ala)_3$-p-nitroanilide, α-N-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA), 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma Chemical Co, St. Louis, MO; styrene oxide (97%), propylene oxide (99%), 1,2-dibromoethane (99%) and 1,2-dichloroethane (99%) were purchased from Aldrich Chemical Co, Inc, Milwaukee, WI. Ethylene oxide (98%) was purchased from Matheson Co, LaPorte, TX. Human plasma was obtained from John Sealy Hospital Blood Bank, The University of Texas Medical Branch, Galveston, Texas.

Incubation of Plasma and α_1-Proteinase Inhibitor with Epoxides and Dihaloethanes

Styrene oxide and propylene oxide solutions were prepared in ethanol (1:1, v/v). 1,2-dibromoethane and 1,2-dichloroethane were prepared in dimethylsulfoxide (1:1, v/v). Ethylene oxide gas was bubbled into a known weight of ethanol and the difference in weight before and after bubbling was the amount of the reagent dissolved in ethanol. Aliquots were added to plasma or α_1-PI solution to the desired concentrations. The reaction mixture was incubated with vigorous shaking at 37°C for 2 hr. Two types of controls were also incubated at 37°C for 2 hr, one containing plasma or α_1-PI in 0.1 M phosphate buffer, pH 7.2 and the other which contained the highest concentration of the particular chemical (in 0.1 M phosphate, pH 7.2) utilized in a given series of experiments. Appropriate aliquots were removed from the incubation mixtures for elastase and trypsin inhibitory assays and for amino group determination, using the TNBS reagent (Habeeb 1966) which specifically reacts with primary amino groups.

Enzyme Assays

Elastase was assayed by the method of Bieth *et al.* (1974). Ten μg of elastase (stock solution, 1 mg per ml of 0.05 M sodium acetate buffer, pH 4.0) in 10 μl, was mixed with 0.2 ml of 0.1 M sodium phosphate buffer, pH 7.2; thereafter, 2.9 ml of the phosphate buffer and 0.1 ml of succinyl-$(Ala)_3$-*p*-nitroanilide (stock solution, 4.5 mg per ml dissolved in dimethylsulfoxide) were added. The rate of change in absorbance at 410 nm was monitored on a Gilford Automatic Spectrophotometer (Model 250) at 25°C.

Trypsin was determined according to the procedure of Erlanger *et al.* (1961). Twenty five μg of trypsin (stock solution, 1 mg per ml of 0.0025 M HCl, pH 3.0) in 25 μl was added to 0.2 ml of 0.1 M sodium phosphate buffer, pH 7.2. Three ml of 0.5 mM μ-N-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) in 0.1 M sodium phosphate buffer, pH 7.2 also containing 0.005 M $CaCl_2$ was added, and the rate of change of absorbance at 410 nm was monitored as described for elastase.

Enzyme Inhibitory Assays

The reaction mixture for the elastase inhibitory assay consisted of samples containing native or chemically treated plasma 10 μl, a native or modified α_1-PI preparation (60 μg, stock solution, 1 mg ml in 0.1 M sodium phosphate buffer, pH 7.2), 10 μg of elastase, and sufficient 0.1 M sodium phosphate, pH 7.2 to bring the volume to 0.2 ml. The mixture was incubated at room temperature for 15 min followed by the addition of 2.9 ml of the sodium phosphate buffer and 0.1 ml of succinyl-$(Ala)_3$-*p*-nitroanilide substrate. The elastase (residual) activity was measured as described above.

The conditions for the trypsin inhibitory assays are the same as those for elastase, except for the substitution of appropriate amount of the enzyme (25μg), inhibitor (60μg), plasma (25μl), and substrate (3.0 ml of 0.5mM BAPNA).

Quantitation of Free Amino Groups with 2,4,6-Trinitrobenzenesulfonic Acid

The quantitation of free amino groups with 2,4,6-trinitrobenzenesulfonic acid (TNBS) was performed by the method of Habeeb (1966). One ml of 0.1% TNBS in water and 1 ml of 4% $NaHCO_3$, pH 8.5 were added to a 1 ml aliquot of an α_1-PI, (1 mg/ml) solution in 4% $NaHCO_3$. The reaction was allowed to take place at 40°C for 2 hr. One ml of 10% sodium dodecyl sulfate (SDS) followed by 0.5 ml of 1 N HCl was added to each sample and the absorbance was read at 335 nm against a blank containing water instead of protein solution and treated as above. Glycine-HCl ranging from 0 to 0.40 μmole per assay was utilized as standard. The decrease in the absorbance values between the control and the experimental incubations was utilized to calculate the number of modified amino groups.

Results

Inactivation of the Proteinase Inhibitory Acitivities of α_1-Proteinase Inhibitor and of Plasma by Ethylene Oxide, Styrene Oxide, and Propylene Oxide

When α_1-PI was incubated at 37°C with increasing concentrations of styrene oxide for 2 hr, both elastase inhibitory capacity (EIC) and trypsin inhibitory capacity (TIC) were reduced. As shown in Figure 1A at 100 mM concentrations of styrene oxide, more than 90% of both TIC and EIC of α_1-PI

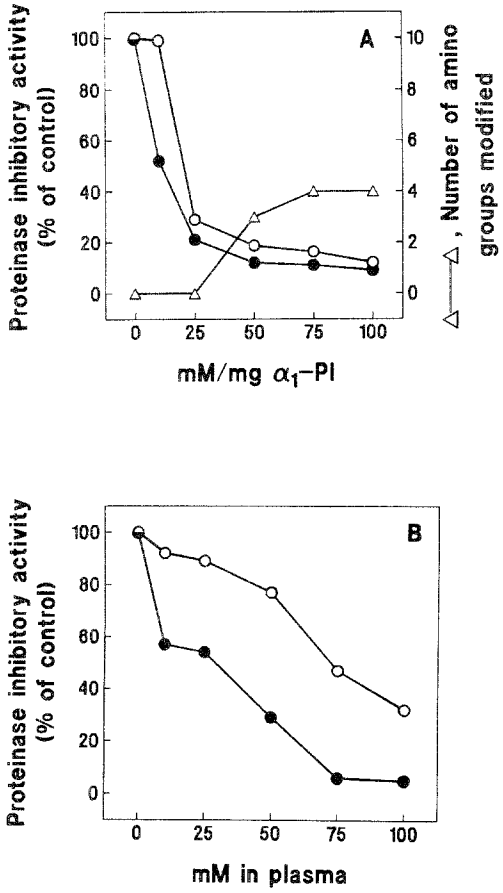

Fig. 1. Inactivation of the proteinase inhibitory activities of α_1-PI or whole plasma when incubated with increasing concentrations of styrene oxide at 37°C for 2 hr. (A) Disappearance of the EIC (●——●) and the TIC (○——○); number of amino groups modified (△——△) as measured by TNBS. (B) Loss of the EIC (●——●) and the TIC (○——○) of whole plasma after incubation with varying concentrations of styrene oxide at 37°C for 2 hr. Each point in this Figure, and in subsequent ones, is the mean of at least three closely agreeing values with different preparations (SD $< \pm 5.7$)

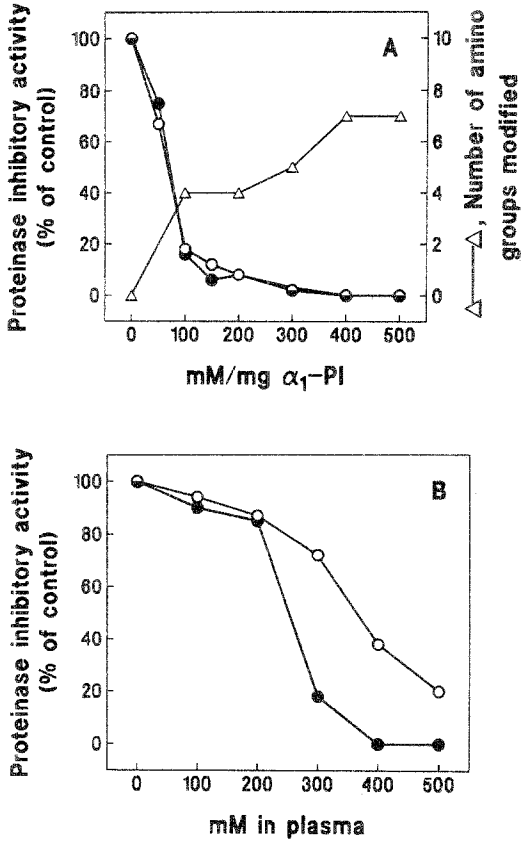

Fig. 2. Inactivation of the proteinase inhibitory activities of α_1-PI or plasma when incubated with increasing amounts of ethylene oxide at 37°C for 2 hr. (A) Disappearance of the EIC (●——●) and of the TIC (○——○) of α_1-PI; (△——△), number of amino groups modified in α_1-PI. (B) Loss of the EIC (●——●) and of the TIC (○——○) of whole plasma (SD $< \pm 6.8$)

were lost. The rate of inactivation of the inhibitory activity was faster at the lower concentration of the styrene oxide. As can be seen in Figure 1A, as many as 4 amino groups in α_1-PI were modified by styrene oxide as determined by the TNBS method. Apparently, the reaction of the lysine (ε-amino groups) residues most likely caused the loss of enzyme inhibitory activities of α_1-PI.

Although no amino group analyses were performed, similar results were obtained when whole plasma was incubated with styrene oxide (Figure 1B). The decrease of the proteinase inhibitory activities in plasma (Figure 1B), however, was more gradual than that of purified α_1-PI (Figure 1A) and the EIC was inhibited more than the TIC.

Ethylene oxide is as effective as styrene oxide in inactivating the proteinase inhibitory activities of α_1-PI (Figure 2A). Thus, when the inhibitor was incubated with 100 mM of ethylene oxide at 37°C for 2 hr, about 80% of both the TIC and EIC of α-PI were lost (Figure 2A). However, when inactivating the protein inhibitory activities of whole plasma, ethylene oxide displayed only about a 25% potency as compared to styrene oxide. As shown in Figure 2B, it requires four times (400 mM) as much ethylene oxide to inactivate the proteinase inhibitory activities of plasma to the same extent as styrene oxide (100 mM, Figure 1B). It is of interest to note that in order for ethylene oxide to effect maximum loss of proteinase inhibitory activities in α_1-PI, seven lysine residues (Figure 2A) have to be modified instead of only 4 in the case of styrene oxide (Figure 1A). Moreover, when 4 lysine residues are modified by ethylene oxide (Figure 2A) there is an

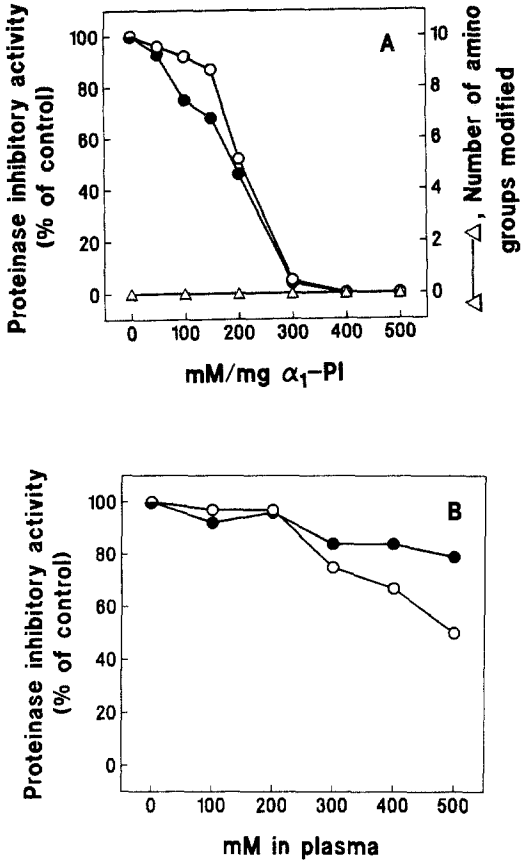

Fig. 3. Inactivation of the proteinase inhibitory activities of α_1-PI or whole plasma during the reaction with increasing concentrations of propylene oxide at 37°C for 2 hr. (A) Disappearance of the EIC (●——●) and the TIC (○——○) of α_1-PI; (△——△), number of amino groups modified per molecule of α_1-PI. (B) Loss of the EIC (●——●) and of the TIC (○——○) of whole plasma (SD $< \pm 3.5$)

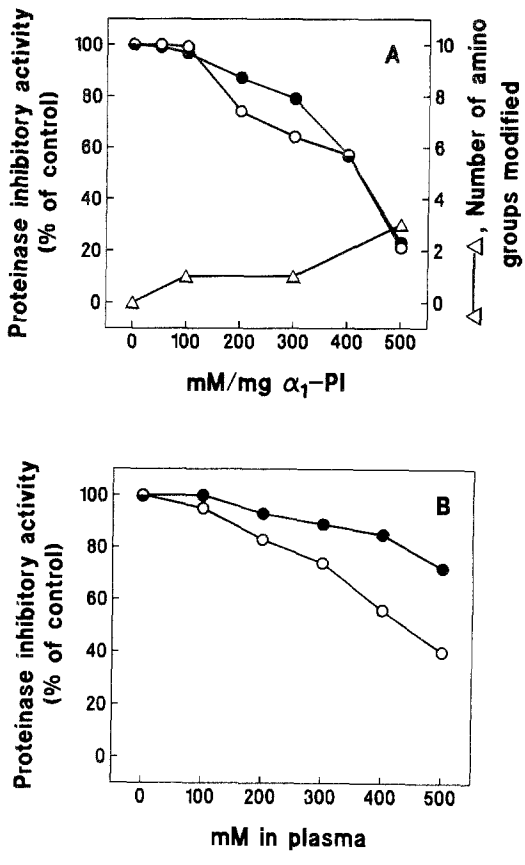

Fig. 4. Inactivation of the proteinase inhibitory activities of α_1-PI or whole plasma during the reaction with varying amounts of 1,2-DCE at 37°C for 2 hr. (A) Disappearance of the EIC (●——●) and of the TIC (○——○) of α_1-PI; (△——△), number of amino groups modified or α_1-PI molecule. (B) Loss of the EIC (●——●) and TIC (○——○) of whole plasma (SD $< \pm 4.5$)

85% decrease in the EIC and TIC of α_1-PI. At higher concentrations (400–500 mM), ethylene oxide is effective in inactivating about 90% of the proteinase inhibitory capacities of whole plasma (Figure 2B) while the same extent of inactivation for α_1-PI is achieved by about 200 mM ethylene oxide (Figure 2A).

Propylene oxide is the least potent of the oxides utilized in the present experiments for inactivating the proteinase inhibitory activities of α_1-PI or whole plasma. Thus, at 100 mM concentration of the reagent, only a 25% and 10% loss of EIC and TIC (Figure 3A), respectively, were observed. This is not surprising, since none of the lysine residues reacted with propylene oxide (Figure 3A), even at the much higher concentrations (300 –500 mM) of the chemicals. The loss of proteinase inhibitory activities could simply be due to physical denaturation of the protein at high levels of the chemical. With plasma at 500 mM concentration of propylene oxide, the corresponding losses are 25% for EIC and 50% for TIC (Figure 3B). Moreover, the inactivation as a function of reagent concentrations is more gradual than those found with ethylene oxide or styrene oxide.

Inactivation of the Proteinase Inhibitory Activities of α_1-Proteinase Inhibitor and of Plasma by 1,2-Dichloroethane or 1,2-Dibromoethane

1,2-Dichloroethane (DCE) (Figure 4A) is less effective in inactivating α_1-PI than any of the epoxides used in present studies. Hence, at 500 mM DCE approximately 30% of the EIC and 25% of the TIC still remain. With the expoxides at 500 mM, (100 mM for styrene oxide) there was an almost complete loss of the proteinase inhibitory activities of

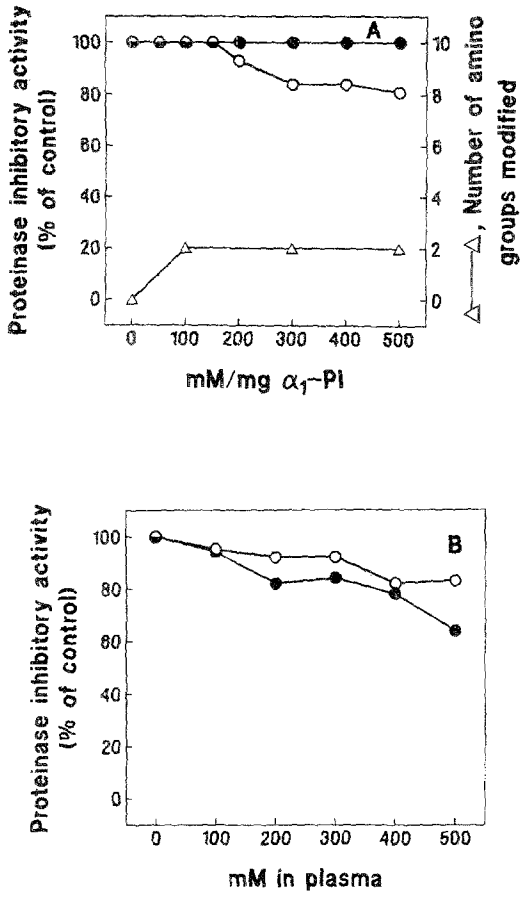

Fig. 5. Inactivation of the proteinase inhibitory activities of α_1-PI during incubation with increasing concentrations of 1,2-DBE at 37°C for 2 hr. (A) Disappearance of the EIC (●——●) and of the TIC (○——○) of α_1-PI; (△——△), number of amino groups modified per molecule of α_1-PI. (B) Disappearance of the whole plasma's EIC (●——●) and TIC (○——○) (SD $< \pm 5$)

α_1-PI (Figures 1A, 2A, 3A). A maximum of 3 amino groups in α_1-PI were alkylated by DCE (Figure 4A).

DCE is as effective in inactivating unfractionated or whole plasma (Figure 4B) as propylene oxide (Figure 3B). Thus, a 30% and 60% reduction of EIC and TIC, respectively, are observed when plasma was incubated with 500 mM DCE (Figure 4B); in comparison, about 20% of the EIC and 50% of the TIC of plasma are abolished with propylene oxide (Figure 3B).

The results obtained with 1,2-dibromoethane (DBE) with α_1-PI (Figure 5A) are different from those observed in the corresponding experiments with DCE (Figure 4A). Although DBE modified 2 amino groups in α_1-PI, it has no effect on EIC, and only a 20% reduction of TIC of α_1-PI (Figure 5A). On the other hand, the inactivating effect of DBE on the EIC of whole plasma (Figure 5B) is not significantly different than that observed with DCE (Figure 4B), but the TIC was reduced less than 20% by DBE (Figure 5B) as opposed to 60% by DCE (Figure 4B).

Discussion

In the present work, we studied individually the effects of styrene oxide, propylene oxide, ethylene oxide, 1,2-dichloroethane (DCE), and 1,2-dibromoethane (DBE) on the proteinase inhibitory activities of α_1-PI and human plasma *in vitro*. All the chemicals, used individually, inactivated either the EIC or TIC of α_1-PI or whole plasma.

Styrene oxide is the most potent inactivator of the proteinase inhibitory activity of α_1-PI and plasma, followed by ethylene oxide and propylene oxide in a decreasing order of potency. Among the epoxides studies, it is interesting to note that propylene oxide does not modify amino groups of α_1-PI. The electron-donating property of the methyl group will reduce the electrophilicity of the oxirane carbon and thus may be preventing the reaction with amino groups of α_1-PI. DCE and DBE are less effective than epoxides in inhibiting α_1-PI.

Since all these compounds are known alkylating agents (Maiorino *et al.* 1982; Farmer *et al.* 1984). the functional groups of the amino acids in proteins they are most likely to modify or react with are: the sulfhydryl of cysteine, the ϵ-amino nitrogen of lysine, the terminal α-amino nitrogen, the imidazole nitrogen of histidine, and probably the hydroxyls of serine and threonine. Derivatives of cysteine and histidine have been isolated from hydrolysates of haemoglobin following exposure to ethylene oxide. The adducts were identified as S-(2-hydroxyethyl) cysteine and N-3-(2-hydroxyethyl) histidine (Ehrenberg *et al.* 1977). 1,2-DBE reacts with the sulfhydryl group of cysteine of albumin (Edwards *et al.* 1970). Hemminki (1986) investigated the binding of styrene oxide to human haemoglobin and serum proteins *in vitro*. This investigator incubated ^{3}[H]-styrene oxide with human blood and purified haemoglobin and serum proteins for amino acid analysis. When the hydrolysates were subjected to separation by high performance liquid chromatography, two radioactive peaks were identified as α-styrene oxide-cysteine and β-styrene oxide—cysteine adducts.

In plasma α_1-PI the only cysteine residue forms an intermolecular disulfide linkage (Jeppson *et al.* 1978) apparently with either cysteine or glutathione and, therefore, no free sulfhydryl group is available for alkylation. Moreover, preparations of α_1-PI

have a blocked amino terminus, and thus unavailable for alkylation (Horng and Gan 1974; Chan *et al.* 1976). Therefore, the most likely functional group to react with these alkylating reagents are the ϵ-amino groups of the lysyl residues. The rate of disappearance of the amino groups is proportional to the concentration of the chemicals. The alkylation of the lysyl residues of α_1-PI may significantly change the conformation of the protein in such a manner that it could no longer bind or interact with the proteinases. We have demonstrated earlier that acetylation of the ϵ-amino groups of the lysine residues of α_1-PI results in the complete loss of inhibitory activity against trypsin and chymotrypsin (Busby and Gan 1976) α_1-PI is the major proteinase inhibitor in human plasma (Heimburger *et al.* 1971) and, therefore, it is safe to assume that the loss of the proteinase inhibitory activity in plasma is due to the modification of α_1-PI.

These *in vitro* results indicate that α_1-PI activity is inhibited in whole plasma at relatively higher concentrations of the chemicals. It is unlikely that humans will be exposed at such high concentrations except in the case of accidental acute exposure. These *in vitro* studies also do not address the effect of metabolites of the chemicals used on the inhibitory activity of α_1-PI, as well as a low dose chronic exposure. Therefore, the effect of chemicals on α_1-PI activity in plasma remains to be evaluated with animals under *in vivo* conditions, as well as detailed population studies on industrial sites, before the efficacy of this method is determined as a useful biological marker of chemical exposure.

Acknowledgments. This investigation was supported in part by grant OH 02149, awarded by the National Institute for Occupational Safety and Health of the Centers for Disease Control.

References

Bieth J, Spies B, Wermuth CG (1974) The syntheses and analytical use of a highly sensitive and convenient substrate of elastase. Biochem Med 11:359–369

Busby TF, Gan JC (1976) Chemical modification of lysyl and arginyl residues of human α_1-antitrypsin. Arch Biochem Biophys 177:552–560

Chan SK, Rees DC, Li SC, Li YT (1976) Linear structure of the oligosaccharide chains in α_1-proteinase inhibitor isolated from human plasma. J Biol Chem 251:471–476

Edwards K, Jackson H, Jones AR (1970) Studies with alkylating esters. II. A chemical interpretation through metabolic studies of the antifertility effects of ethylene dimethanesulfonate and ethylene dibromide. Biochem Pharmacol 19:1783–1789

Ehrenberg L, Osterman-Golkar S, Segerback D, Svensson K, Calleman CJ (1977) Evaluation of genetic risks of alkylating agents. III. Alkylation of haemoglobin after metabolic conversion of ethene to ethene oxide *in vivo*. Mutat Res 45:175–184

Erlanger BF, Kokowsky N, Cohen W (1961) The preparation and properties of two new chromogenic substrates for trypsin. Arch Biochem Biophys 95:271–278

Farmer PB, Bailey E, Campbell JB (1984) Use of alkylated proteins in the monitoring of exposure to alkylating agents, In: Berlin A, Draper M, Hemminki K, Vainio H (eds), Monitoring Human Exposure to Carcinogenic and Mutagenic Agents, Proc Joint Symposium held in Espoo, Finland, Dec. 12–15, IARC Scientific Publications 59:189–198

Gan JC, Ansari GAS (1986) Non-oxidative inactivation of α_1-proteinase inhibitor by carbonyl compounds found in cigarette smoke. Res Commun Subs Abuse 7:59–69

——— (1987) Plausible mechanism of inactivation α_1-proteinase inhibitor by acrolein. Res Commun Chem Path Pharmacol 55:419–422

Habeeb AFSA (1966) Determination of free amino groups by trinitrobenzenesulfonic acid. Anal Biochem 14:328–336

Heimburger N, Haupt H, Schwick HG (1971) Proteinase Inhibitors of Human Plasma. In: Fritz H, Tschesche H (eds). Proc Internl Proteinase Inhibitors, Walter de Gruyter, Berlin

Hemminki K (1986) Covalent binding of styrene oxide to amino acids, human serum proteins and haemoglobin. Prog Clin Biol 207:159–168

Horng WJ, Gan JC (1974) Purification and characterization of human plasma α_1-antitrypsin. Texas Report Biol Med 32:489–504

Jeppson JO, Laurell CB, Fagerhol M (1978) Properties of isolated human α_1-antitrypsins of Pi types M, S and Z. Eur J Biochem 83:143–153

Kirsch-Volders M (ed) (1984) Mutagenicity, Carcinogenicity, and Teragenicity of Industrial Pollutants. Plenum Press, New York

Maiorino RM, Gandolfi AJ, Brendel K, MacDonald JR, Sipes IG (1982) Chromatographic resolution of amino acid adducts of aliphatic halides. Chem Biol Interactions 38:175–188

Marshall T, Vesterberg O (1983) Effect of chemical exposure on rat serum proteins revealed by a modified technique of two-dimensional electrophoresis. Electrophoresis 4:363–366

Parmeggiani T (ed) (1983) Encyclopeadia of Occupational Health and Safety, Third (Revised) Edition, International Labor Organization, Vols. 1 and 2, Geneva, Switzerland

Piafsky KM (1980) Disease-induced changes in the plasma binding of basic drugs. Clin Pharmacol Kinetics 5:246–262

Reish MS (1987) Top 50 chemicals production steadied in 1986. Chem Eng News April 13:20–26

Manuscript received July 6, 1987 and in revised form October 26, 1987.