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SHORT COMMUNICATION

Effects of Inhaled Hexamethylene Diisocyanate (HDI) on Guinea Pig Cholinesterases[1]

Effects of Inhaled Hexamethylene Diisocyanate (HDI) on Guinea Pig Cholinesterases. KAROL, M. H., HANSEN, G. A., AND BROWN, W. E. (1984). *Fundam. Appl. Toxicol.* **4**, 284–287. Hexamethylene diisocyanate, HDI, a starting material in the production of many polyurethane products, was found to inhibit stoichiometrically mammalian and electric eel cholinesterases in an *in vitro* system (W. E. Brown, A. H. Green, M. H. Karol, and Y. Alarie, 1982, *Toxicol. Appl. Pharmacol.* **62**, 45–52). The current study examined *in vivo* effects on guinea pig cholinesterases resulting from inhalation of HDI. Guinea pigs were exposed to atmospheres of 0.5, 1.8, or 4.0 ppm HDI (ceiling value = 0.02 ppm) for up to 6 hr. Blood samples were drawn prior to exposure and at specified times during exposure. No inhibition of serum cholinesterase was detected following exposure to 0.5 ppm HDI for 6 hr, to 1.8 ppm HDI for 2 hr, or to 4.0 ppm HDI for 3 hr. Similarly, no inhibition was detected when erythrocytes from each blood sample were assayed for acetylcholinesterase activity. Last, animals were sacrificed and cholinesterase activity determined in bronchial lavage fluid. Enzyme levels of HDI-exposed animals were not significantly different ($P > 0.05$) from those of control animals exposed to water vapor. In conclusion, although *in vitro* experiments had demonstrated potent anticholinesterase activity by HDI, *in vivo* inhalation exposure of guinea pigs to HDI at concentrations 25–200 times above the recommended (ACGIH) ceiling value did not produce measurable inhibition of cholinesterase activity.

Several adverse health effects have been associated with the increased industrial use of diisocyanates. Such effects include: allergic sensitization of workers (Avery *et al.*, 1969; Pepys *et al.*, 1972; O'Brien *et al.*, 1979; Karol *et al.*, 1979; Zeiss, *et al.*, 1980), chronic respiratory disease and pneumonitis (Charles *et al.*, 1976), and dermal irritation (Bruckner *et al.*, 1968). In experimental animals, irritation of the cornea (Luckenbach and Kielor, 1980) and respiratory tract (Sangha and Alarie, 1979) have been noted. Although most of these reports were concerned with either toluene diisocyanate (TDI) or diphenylmethane diisocyanate (MDI), hexamethylene diisocyanate (HDI) was found to be a potent sensory irritant in mice (Sangha *et al.*, 1981), to cause skin sensitization in guinea pigs (Kondratyev and Mustayev, 1974), and was associated with bronchial hyperreactivity and asthma in automobile spray painters (Karol and Hauth, 1982; Cockcroft, 1982).

Studies were undertaken in this laboratory to investigate mechanisms underlying the respiratory effects of HDI. The various bronchoconstrictor respiratory reactions resulting from inhalation of HDI suggested possible cholinergic involvement. Using cholinesterases from both mammalian and electroplax sources, an *in vitro* study revealed specific interaction of HDI with active sites on these enzymes (Brown *et al.*, 1982a). Enzyme inhibition occurred regardless of whether HDI was added as a liquid or vapor to the *in vitro* system. The apparent specificity of reaction of HDI with the mammalian cholinesterases suggested biological importance for the interaction and a possible mechanism for the sensory and pulmonary irritation observed in workers following HDI inhalation. The current study evaluated cholinesterase activity in both blood and respiratory tract secretions of guinea pigs following inhalation of HDI vapor.

METHODS

Animals. Male, English smooth-haired guinea pigs were obtained from Hilltop Laboratories, Scottdale, Pennsylvania.

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Exposure to HDI vapor. Animals were placed in body plethysmographs which were connected to a 10-liter Plexiglas inhalation chamber described previously (Karol *et al.*, 1978). Only the heads of animals extended into the chamber.

HDI vapor was generated by bubbling dried air through the isocyanate (Mondur HX, Mobay Chemical Corp., 98%) contained in a glass impinger (Sangha *et al.*, 1981). Vapors from the impinger were diluted with dried room air and drawn into the inhalation chamber which was operated under a constant airflow of 20 liters/min. The concentration of HDI in the exposure chamber was determined by sampling the chamber during animal exposures and analysis by gas chromatography as described (Sangha *et al.*, 1981).

Control animals were exposed in an identical manner to water vapor generated by placing distilled water, rather than HDI, in the impinger.

Cholinesterase assays. Blood was collected from the nailbed of guinea pigs prior to and during HDI exposures. Red blood cells were collected by centrifugation for 15 sec at 9000*g* in a Beckman microfuge (Model 152, Beckman Instruments, Fullerton, Calif.) and acetylcholinesterase activity determined according to Ellman *et al.* (1961) using acetylthiocholine iodide (Sigma Chemical Co., St. Louis, Mo.) as substrate. Serum cholinesterase activity was determined using butyrylthiocholine chloride (Sigma) as substrate.

Lung lavage. Following HDI exposure, animals were killed by an overdose of Nembutal (Abbott Laboratories, North Chicago, Ill.). Lung lavage was obtained by injection into the trachea of 10 ml warmed saline. The fluid was withdrawn, then centrifuged and the supernatant evaluated for cholinesterase activity using butyrylthiocholine chloride substrate. The protein content of the lavage was measured using the Bio-Rad protein reagent (Bio-Rad Laboratories, Richmond, Calif.).

Statistics. All results were evaluated for statistical significance using Student's *t* test.

RESULTS

Effects of HDI on erythrocyte acetylcholinesterase (AChE) and serum cholinesterase (ChE). The concentrations of HDI, lengths of exposure, and size of exposure groups, are listed in Table 1. No significant enzyme inhibition ($p < 0.05$) was detected when enzyme activity of individual animals following exposure was compared with values obtained immediately prior to HDI exposure.

Animals exposed to 1.8 ppm HDI displayed severe respiratory irritation during exposure as evidenced by a slowed respiratory rate and labored breathing. Of the four guinea pigs exposed to 4.0 ppm HDI, two animals died within 1 hr and the surviving animals showed severe respiratory distress. Yet, AChE and ChE activities in surviving animals were unchanged when compared with preexposure values.

TABLE 1

SUMMARY OF EXPERIMENTAL HDI EXPOSURES AND CHOLINESTERASE MEASUREMENTS

HDI exposures	
0.5 ppm for 6 hr ($n = 6$)	
1.8 ppm for 2 hr ($n = 4$)	
4.0 ppm for 3 hr ($n = 4$)[a]	
Cholinesterase determinations	
Erythrocyte acetylcholinesterase	
Serum cholinesterase	
Bronchial lavage fluid cholinesterase	

[a] Two of the four guinea pigs died during the first hour of exposure to 4.0 ppm HDI.

Effect of HDI on pulmonary ChE. Immediately following exposures to HDI, animals were killed and lung lavage taken. ChE activity (micromoles butyrylthiocholine chloride hydrolyzed per minute per milligram of protein) in lavage fluid of HDI-exposed animals was compared with activity in lavage from control animals. Statistically, there was no difference between the values ($p > 0.05$). Enzymic activity in pulmonary lavage could not be determined for animals surviving exposure to 4.0 ppm HDI because lungs from these animals were too severely hemorrhaged to obtain a lavage.

DISCUSSION

Serum and red cell cholinesterase activities have been employed for biologic monitoring of exposure to organophosphate insecticides. Changes in cholinesterase activities in the blood of man and animals as a result of exposure to numerous enzyme inhibitors have been extensively reviewed by Wills (1972). Manno and Lotti (1976) investigated serum

and red cell cholinesterase activities in 30 workers exposed to toluene diisocyanate. These authors reported a decreased AChE activity in 70% of the cases when the exposed group was compared with a control group. No difference in serum ChE levels between groups was noted. The exposure concentrations of TDI were "negligible" when measured according to Marcali (1957).

We have previously shown that HDI had potent anticholinesterase activity in an *in vitro* system (Brown *et al.*, 1982a). Enzyme inhibition was observed regardless of the source of enzyme (eel or mammalian), its purity, or whether HDI was introduced as a liquid or vapor. This potent stoichiometric inhibition of enzymatic activity was postulated as a possible mechanism for the bronchoconstrictive respiratory effects which result from inhalation of HDI.

The current study explored *in vivo* cholinesterase inhibition by HDI examining both the erythrocyte and serum enzymes. Guinea pigs were exposed to HDI concentrations ranging from 0.5 to 4.0 ppm. The latter concentration was extremely toxic to all animals and lethal within 1 hr for half of the animals. No inhibition of AChE or ChE activity was detected even at these elevated HDI concentrations. Moreover, when bronchial lavage was assayed to determine possible inhibition of localized enzyme, no decrease in ChE activity was detected following exposure to 1.8 ppm HDI.

Several explanations may be offered for the above results: First, HDI, possessing reactive isocyanate groups may not have reached enzyme sites. Rather, HDI may have been hydrolyzed either in the exposure chamber before inhalation, or within the guinea pig respiratory tract. However, evidence refuting this possibility was obtained. Severe sensory and pulmonary irritation was noted in animals exposed to HDI. Irritation of this nature meant that HDI was present as the isocyanate, not as the hydrolyzed hexamethylene diamine product. Moreover, in previous studies (Brown *et al.*, 1982a) *in vitro* inhibition of ChE activity occurred when the enzyme was dissolved in water and HDI introduced as a vapor. Under those conditions, HDI was able to inactivate the aqueous enzyme solution in less than 1 min demonstrating a strong affinity of the isocyanate for the enzyme even in the presence of water.

A second possible explanation for lack of *in vivo* anti-ChE activity by HDI may be the failure of HDI to reach the blood stream where enzymes were measured. In agreement with this finding is the report that carbamate inactivation of ChE was detected only when the inhibitors were injected intravenously but not when administered ip or po (Vandekar *et al.*, 1971). However, we did not attempt intravenous administration of HDI since the intent of our study was to investigate mechanisms of toxicity resulting from inhalation of HDI.

Third, enzyme inhibition may not have been apparent because of the reversibility of the HDI–ChE interaction (Brown *et al.* 1982b). Studies in this laboratory have shown the half-life of reactivation *in vitro* at pH 7.5 was 3.5 hr. Although in the current study all enzyme assays were performed within 1 hr of HDI exposure, the *in vivo* reactivation may have a shorter half-life dependent upon the local nucleophilic environment. Therefore, enzyme reactivation may have occurred before blood (and lung) samples were taken.

Last, consideration should be given to both the amount of enzyme in the blood stream and respiratory tract, and to the presence of acetylcholine receptor in the lung. The amount of enzyme present in blood and lung tissue may have been so high that any enzyme inactivation was insignificant in comparison to the total amount of enzyme present. Similarly, enzyme inhibition might not have been detected because of reaction of HDI with acetylcholine receptor as well as with soluble enzyme. In this case, the hydrophobic nature of HDI, may have led to preferred interaction with membrane bound receptor rather than with soluble enzyme. Under these conditions, enzyme inhibition would not have been apparent, although, as suggested by Wills (1972),

it may be that only cholinesterase inhibition *at the site* of neuroeffector release has any absolute functional significance.

In summary, although HDI had been shown to be a potent ChE inhibitor, *in vitro,* no enzyme inhibition was detected in guinea pigs exposed by inhalation to HDI concentrations ranging from 0.5 to 4.0 ppm.

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