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Covalent Interactions of Toluenediisocyanate with DNA and Proteins

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ABSTRACT *: The covalent interactions of toluenediisocyanate (TDI) with macromolecules were investigated both in vitro and in vivo. In vitro incubations of 2,4- and 2,6-TDI with DNA or proteins resulted in dose-dependent formation of TDI-protein and TDI-DNA adducts. TDI-treated DNA was highly resistant to enzymatic digestion and thermal hydrolysis, but was readily hydrolyzed under acidic conditions by releasing its corresponding toluenediamine (TDA), suggesting that TDI caused the crosslinking of DNA. Reaction of TDI with albumin and globin resulted in the formation of several adducts, and some adducts were formed in blood of TDI-treated rats in a dose-dependent fashion. Administration of TDI to rats resulted also in a dose-dependent binding of TDI to hepatic tissue. Levels of TDI-albumin adducts were 10 times higher than those of TDI-globin adducts; the biological half lives of TDI-albumin and TDI-globin adducts were 1.2 and 12.5 days, respectively. Globin adducts were detected up to 28 days after the treatment. Hepatic TDI protein adducts were persistent for a substantial period whereas the levels of hepatic TDI-DNA adduct were decreased rapidly. These results indicate that the isocyanato group of TDI is not readily hydrolyzed under physiological conditions, is transported to other organs, and is bound to DNA and/or proteins without further metabolic activation. As the adducted products degrade in the body, TDA is released and introduced to the liver. TDA may additionally bind to hepatic tissue after metabolic activation. Thus, the toxic effect of TDI exposure is considered to persist during the lifetime of the adducted biological macromolecules.*

Key Words *: Toluenediisocyanate, Toluenediamine, DNA binding, Protein adduct*

I. INTRODUCTION

Toluene diisocyanate (TDI) is one of the most widely-used industrial chemicals which causes mucosal irritation (Stepano *et al.*, 1993). Such irritation on the pulmonary system by inhaled TDI has been considered to induce various respiratory diseases including occupational asthma. Animal experiments have shown the production of TDI-specific hypersensitivity antibodies and their relation to respiratory responsiveness (Karol *et al.*, 1980; Karol, 1983). However, the pathogenesis of TDI sensitization and its initial molecular reactions are not well-understood in spite of many lines of investigation.

In addition to pulmonary irritation and immunological sensitization, the carcinogenic potential of TDI has been of great interest due to the formation of diamines under physiological conditions, eventhough TDI itself is known to be noncarcinogenic when inhaled (Loeser, 1981; Dieter *et al.*, 1990; Doe and Hoffman, 1995). Toluenediamine (TDA), the product of aqueous hydrolysis of TDI, has proven to be mutagenic in bacterial models and is carcinogenic in rodents (Hayward *et al.*, 1995). In addition, TDI has been reported to induce single- and double-strand breaks in the DNA of white blood cells *in vitro* and is also considered to cause crosslinking of DNA (Merczynski *et al.*, 1992). TDA has been detected and quantitated in hydrolyzed plasma and urine samples from TDI-exposed workers in a dose-related fashion, which can be a useful method in biomonitoring (Rosenberg and Savolainen,

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ABBREVIATIONS
TDI, Toluenediisocyanate; TDA, Toluenediamine; GC-MS, Gas Chromatography Mass Spectrometry; HPLC, High Performance Liquid Chromatography; PFPA, Pentafluoropropionic Anhydride.

1986).

Studies using ^{14}C-labeled TDI have revealed that the radioactivity from the inhaled substrate is rapidly absorbed into the bloodstream and excreted through the urine. However complete clearance of the radioactivity has been reported to last for two weeks (Kennedy *et al.*, 1989, 1994). Most of the radioactivity in the blood was found in the plasma as albumin or other protein conjugates with the remaining amount in the erythrocytes (Kennedy *et al.*, 1994). A recent investigation on the characterization of TDI-hemoglobin adducts of guinea pigs exposed to TDI vapor, employing immunochemical recognition together with ionspray mass spectroscopy, demonstrated that two types of adducts are formed: carbamoylation products, and amine-nitroso adducts. The adduction procedure is thus considered to involve both direct reaction of isocyanato group with biological nucleophiles and reaction of amine moiety resulting from aqueous hydrolysis of isocyanto group with hemoglobin (Jin *et al.*, 1993; Day *et al.*, 1996). However little or no information is available regarding the pathways involved in the reaction of TDI with DNA and the identity of TDI-DNA adducts.

In the present investigation, we intended to provide further evidence for protein and DNA cross-linking ability of TDI under physiological conditions as well as the pathways of toxic effects raised from such damage. Incorporation of TDI molecule into DNA and/or blood proteins is considered to cause prolonged toxicity by releasing TDA continuously as the adducted products degrade in the body.

II. MATERIALS AND METHODS

1. Materials

2,4-TDI, 2,6-TDI, technical grade TDI (4 : 1 mixture of 2,4- and 2,6-TDI), 2,4-TDA, 2,6-TDA, and pentafluoropropionic anhydride (PFPA) were obtained from Aldrich Chemical Co. (Milwaukee, WI). d^3-2,4-TDA was obtained from Synthelec (Sweden). Biochemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were highest grade commercially available.

2. *In vitro* Reaction of TDI with Proteins

Rat serum albumin (10 mg) and globin (10 mg) in 1 *ml* of 10 mM KH_2PO_4 buffer (pH 7.4) were incubated with 0.15, 1.5, 15, and 150 MM of 2,4- or 2,6-TDI for 1 hour at 37°C. Albumin was precipitated by the addition of 10 *ml* cold acetonitrile followed by centrifugation. The resulting precipitates were sequentially rinsed with acetonitrile, ethyl alcohol, and ethyl ether for 3 times and dried under nitrogen stream. In case of globin, reaction mixtures were poured into 5 *ml* of 50 mM HCl in propanol and globin was precipitated by ethyl acetate. The precipitates were washed three times with ethyl acetate to remove heme and unbound TDI. The pellets were further rinsed and dried as described for albumin.

For the identification of protein adducts, dried albumin or globin (5 mg) were dissolved in 1 *ml* of 50 mM Tris-HCl buffer (pH 7.5) and treated with 150 units of Pronase E for 20 hours at 37°C. Incubation mixtures were directly analyzed by HPLC. A portion of TDI-treated rat albumin or globin was dissolved in 0.5 *ml* of 6 N HCl and heated for 2 h at 100°C. The hydrolysates were neutralized by addition of K_2CO_3/$NaHCO_3$ (2/1) mixture, and then extracted with ethyl acetate (2×2 *ml*). The extracts were dried under nitrogen stream and dissolved in 0.1 *ml* of acetonitrile for HPLC analysis. HPLC analysis was conducted with a Supelco-C18-DB column (5 mm, 0.46×25 cm). The column was eluted at a flow rate of 1.0 *ml*/min with 10% CH_3CN (v/v) in 0.1% trifluoroacetic acid solution with a linear gradient to 70% CH_3CN over 30 min and the peak was monitored at 254 nm.

3. *In vitro* reaction of TDI with DNA

Calf thymus DNA (4 mg) dissolved in 1 *ml* of 20 mM KH_2PO_4 buffer (pH 7.4) was incubated with 0.03, 0.3, 3, 30, and 300 MM of 2,4- or 2,6-TDI for 20 hours at 37°C. Unreacted TDI or its polymeric material was removed by extraction with phenol/chloroform/isoamyl alcohol (25/24/1) and with chloroform/isoamyl alcohol (24/1). DNA was precipitated by addition of 2 volumes cold ethanol, and rinsed 3 times with ethanol. After brief drying,

the DNA was dissolved in 2 *ml* of 10 mM tris-HCl/ 5 mM $MgCl_2$ (pH 7.0) and digested as follows: Micrococcal Nuclease (200 units, 30 min), Nuclease S1 (300 units, 1 hour), Nuclease P1 (20 units, 1 hour). The hydrolysates were then analyzed by using reverse phase HPLC column (Supelcosil C18, 5 mm, 0.46×25 cm). The column was eluted at a flow rate of 1.2 *ml*/min with 5% CH_3CN in 20 mM NH_4OAc (pH 5.5) for 5 min followed by a linear gradient to 30% CH_3CN over 20 min. The eluate was monitored at 254 nm.

4. Acidic and Thermal Hydrolysis of DNA

Aliquots of the DNA solution were subjected to acidic or thermal hydrolysis by heating for 2 hours at 90°C with or without the addtion of an equal volume of 1 N HCl. The hydrolysates were analyzed by HPLC after neutralization if necessary.

5. Animal Treatments

Male Sprague-Dawley rats (220~225 g) were obtained from Daihan Laboratory Animal Co. (Seoul, Korea) and were housed in polycarbonate cages with sterilized hardwood chip bedding and filter top. Throughout the study, animals were allowed food and water ad libitum. Rats, in groups of three, were given TDI solution in dry hexane by i.p. injection at a dose of 10, 20, and 40 mg/kg body wt. Animals were sacrificed 1, 2, 4, 7, 14, and 28 days after the treatment. Blood samples were collected in EDTA-containing vacutainers by a cardiac puncture and the liver was isolated and frozen immediately.

6. Isolation of Globin and Albumin

Albumin was separated using the method of Skipper *et al.* (1985). Briefly, blood was centrifuged at 2000 g to separate red blood cells and plasma. An equal volume of saturated ammonium sulfate was added dropwise to plasma and samples were centrifuged at 9000 g for 15 min. The supernatant was removed and the pH was adjusted to 5 with 1 N acetic acid. Samples were centrifuged at 9000 g for 10 min and precipitate was dissolved in 50 mM Na_2HPO_4, pH 7.4. Albumin was precipitated by cold acetonitrile and washed as described above.

Globin was isolated from blood pellet by the methods described by Ascoli *et al.* (1981). The resulting globin precipitate was washed with pure acetonitrile and ethyl ether to remove unbound TDI or TDA.

7. Isolation of Hepatic DNA and Protein

Hepatic DNA and protein was isolated by slightly modified method described by Gupta (1984) and Kinoshita and Gelboin (1972). Frozen liver tissue (1 g) was thawed in 10 *ml* of 1% SDS/1 mM EDTA and homogenized. The homogenate was mixed with 0.5 *ml* of 1.0 M Tris-HCl (pH 7.4) and extracted with 10 *ml* of equilibrated phenol followed by 10 *ml* of chloroform/isoamyl alcohol (24/1). The aqueous layer was mixed with 1 *ml* of 5 M NaCl and DNA was precipitated by addition of 10 *ml* cold ethanol. The DNA lump was washed with 70% ethanol and reconstituted in 2 *ml* of 50 mM tris-HCl (pH 7.4). Sample solution was incubated with RNase A (60 g/ *ml*) and RNase T1 (200 U) to degrade RNA. The mixture was mixed with 0.5 *ml* of 1.0 M Tris-HCl (pH 7.4) and extracted with 10 *ml* of equilibrated phenol followed by 10 *ml* of chloroform/isoamyl alcohol (24/1). The aqueous layer was extracted and washed according above procedures. The resulting DNA was reconstituted in 50 mM tris-HCl (pH 7.4) for further study. Protein was precipitated in phenol layer by addition of 10 volume of cold acetonitrile and washed six times with acetonitrile and ethyl ehter to remove unbound TDI or its surrogates.

8. Measurement of TDA Released from Macromolecules

Measurement of TDA released from protein or DNA adducts was done by the modified method described by Skarping *et al.* (1994). Briefly, 2~5 mg of protein or DNA was dissolved in 1.5 *ml* of 6 N HCl containing 0.1 ng trideuterated 2,4-TDA and hydrolysed at 100°C for 8 hours. The hydrolysates were then alkalized with 5 *ml* of saturated NaOH, extracted twice with 4 *ml* of toluene. The extracted TDA was derivatized with 20μ*l* of PFPA reagent.

After the derivatization, excess PFPA was removed by extracting with 4 m*l* of 1 M K_2HPO_4 (pH 7.4). Organic layers were concentrated to 70 μ*l* using vacuum evaporator and analyzed by GC-MS. GC-MS analysis was done on a HP 5972 mass selective detector coupled to HP 5890 Series II GC equipped with HP 7623 auto sampler. HP-5 (20 m × 0.25 μm i.d., 0.25 μm film thickness) capillary column was interfaced directly with MS source. Mass spectrometric data were obtained in selected-ion mode (SIM) for m/z 295 and 414 to monitor TDA-PFPA derivatives and for m/z 298 and 417 to monitor trideuterated TDA-PFPA derivatives. Calibration plots for globin and albumin spiked with 2,4- and 2,6-TDA were linear over the concentrations of 0.1∼50 pmol/mg protein.

III. RESULTS

1. Quantitation of TDA Released from DNA and Protein

Quantitation of TDA in acid hydrolyzed DNA or proteins was done after derivatization with PFPA. Fig. 1 shows mass selective chromatogam of 2,4-, 2,6- and d_3-2,4-TDA-PFPA. The fragment ions at m/z 295 and 414 were chosen for TDA-PFPA derivatives, and at m/z 298 and 417 for trideuterated TDA-PFPA derivatives. The detection limit was 0.05 pmol TDA/mg macromolecules.

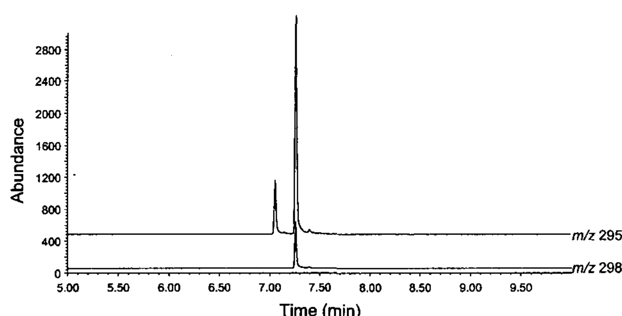

Fig. 1. Multiple ion detection mass spectral trace (m/z 295 and m/z 298) for the analysis of 2,4-TDA-PFPA (7.27 min), 2,6-TDA-PFPA (7.08 min), and trideuterated 2,4-TDA-PFPA derivatives (7.27 min).

2. In vitro TDI-macromolecular Adducts

The levels of binding were proportional to concentrations over the range of 0.15∼150 mM TDI without saturation (Table 1). The order of amounts of TDI binding was BSA> globin> DNA. Binding of TDI to DNA was much less than that to BSA or globin. There was no obvious difference in the binding affinity of 2,4- and 2,6-TDI isomer to macromolecules.

Enzymatic digestion of the treated proteins by pronase E showed several adducts as monitored by UV at 254 nm. The comparison of the chromatograms of TDI-treated albumin and globin with solvent control is illustrated in Fig. 2. In case of albumin, the peaks at 53 and 54 min were considered to be the major adducts, while the major adduct peaks of globin were eluted at 46 and 47 min. These adducts were disappeared upon acidic hydrolysis producing TDA (chromatograms not shown), suggesting that the adducts resulted from direct reaction of TDI or partially hydrolyzed TDI with protein bases.

TDI-treated DNA was demonstrated to be highly resistant to enzymatic digestion by sequential application of Micrococcal Nuclease, Nuclease S1, and Nuclease P1, which produces mononucleotides from normal DNA. The HPLC chromatograms of the

Table 1. *The binding of TDI to macromolecules after in vitro incubations of TDI with globin, albumin, and calf thymus DNA*[a]

Concentration (mM)	2,4-TDI adducts (pmol/mg DNA or protin)			2,6-TDI adducts (pmol/mg DNA or protein)		
	albumin	globin	DNA	albumin	globin	DNA
0.15	0.19±0.01[b]	0.20±0.02	0.01±0.01	0.22±0.08	0.07±0.03	nd[c]
1.5	5.84±5.53	0.61±0.11	0.04±0.01	2.65±0.02	0.51±0.04	0.04±0.01
15	26.84±25.03	3.86±1.18	0.12±0.02	19.23±4.97	4.07±1.20	0.10±0.02
150	69.76±37.9	32.2±9.6	1.24±0.04	126.3±86.9	36.03±6.36	0.27±0.02

[a]The adducts were determined by measuring its corresponding TDA released after acid hydrolysis as described under Materials and Methods.
[b]Each value represents the mean±SD of three determination.
[c]not detected.

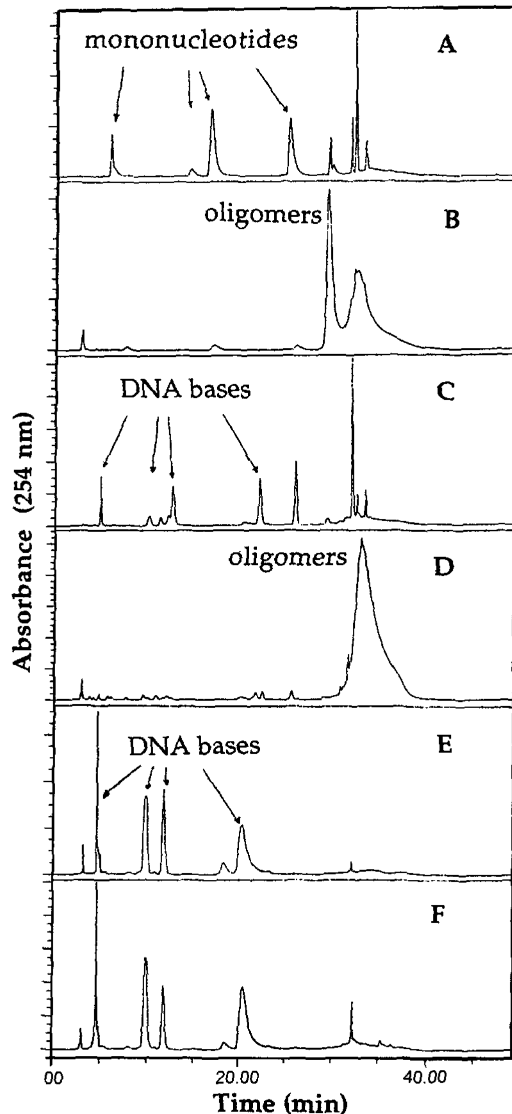

Fig. 2. HPLC chromatograms of TDI-treated albumin and globin and those of control experiment after pronase E digestion. In case of albumin (B), unidentified adducts are observed at 53 and 54 min (shaded peaks) with other minor peaks compared to control (A). Globin (D) showed major adducts at 46 and 47 min (shaded peaks) compared to control (C).

enzymatic hydrolysates of TDI-treated and control DNA are shown in Fig. 3A and 3B. TDI-treated DNA showed peaks largely in oligomer range (28~40 min) with very small amount of mononucleotide production, whereas most of the control DNA hydrolyzed to mononucleotides, which elute before 28 min. Figs. 3C and 3D are the chromatograms of the samples thermally hydrolyzed, while Figs. 3E and 3F are those of acidic hydrolysates. In thermal hydrolysis, TDI-treated DNA showed much less

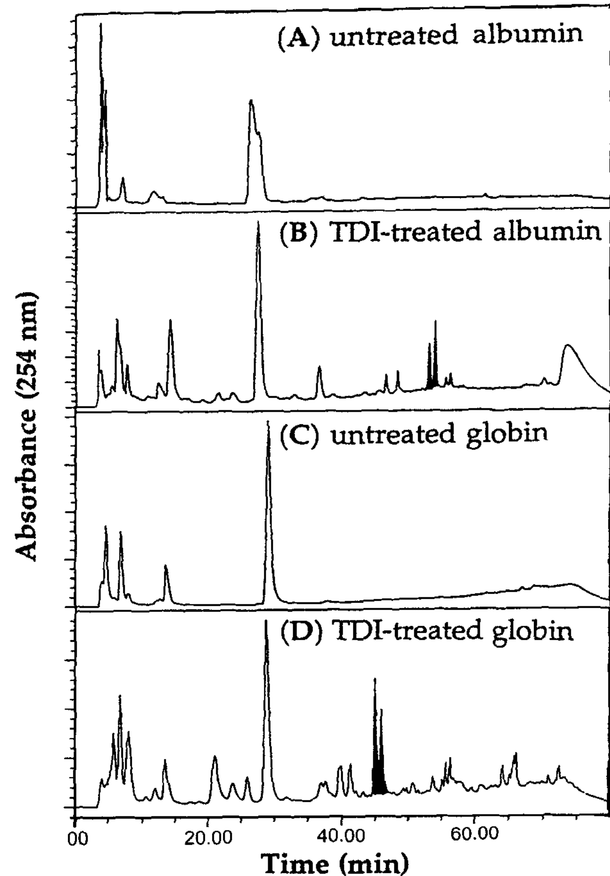

Fig. 3. HPLC chromatograms of TDI-treated calf thymus DNA after enzymatic (A, B), thermal (C, D), and acidic (E, F) hydrolysis. In case of enzymatic (B) or thermal (D) hydrolysis samples, TDI-treated DNA was not hydrolyzed (oligomer peaks at 28~40 min only were observed) to give mononucleotides or DNA bases as observed in the control samples (A, C). Acidic hydrolysis released bases from both the control (E) and the TDI-treated DNA (F).

amount of released bases compared to the control DNA, which indicates heavy crosslinking of DNA by TDI molecule. The oligomer observed both in enzymatic and thermal hydrolysates of TDI-treated DNA were hydrolyzed to produce bases under acidic conditions by releasing TDA. The release of TDA was clearly demonstrated by GC/MS analysis.

3. *In vivo* TDI-blood Protein Adducts

TDI-protein adducts were monitored in blood proteins 1 day after i.p injection of technical grade TDI to rats at a single dose of 10, 20, and 40 mg/kg body wt. Both 2,4- and 2,6-TDI resulted in the binding to albumin and globin in a dose-dependent

Table 2. *In vivo* TDI-adducts formation in blood hemoglobin and albumin following administration of technical grade TDI to rats[a]

Dose (mg/kg)	2,4-TDI adducts (pmol/mg protein)		2,6-TDI adducts (pmol/mg protein)	
	albumin	globin	albumin	globin
10	3.96±1.50[b]	0.19±0.08	2.76±0.84	0.11±0.03
20	10.12±1.11	0.57±0.12	6.58±0.53	0.38±0.05
40	18.40±5.15	1.82±0.41	7.42±3.13	0.92±0.38

[a]TDI was injected i.p. in rats at a single designated dose. Blood was taken 1 day after treatment with TDI and albumin and globin were isolated. The TDI adducts were determined by measuring its corresponding TDA after acid hydrolysis as described under Meterials and Methods.
[b]Each value respresents the mean±SD of determination made with 3~4 rats.

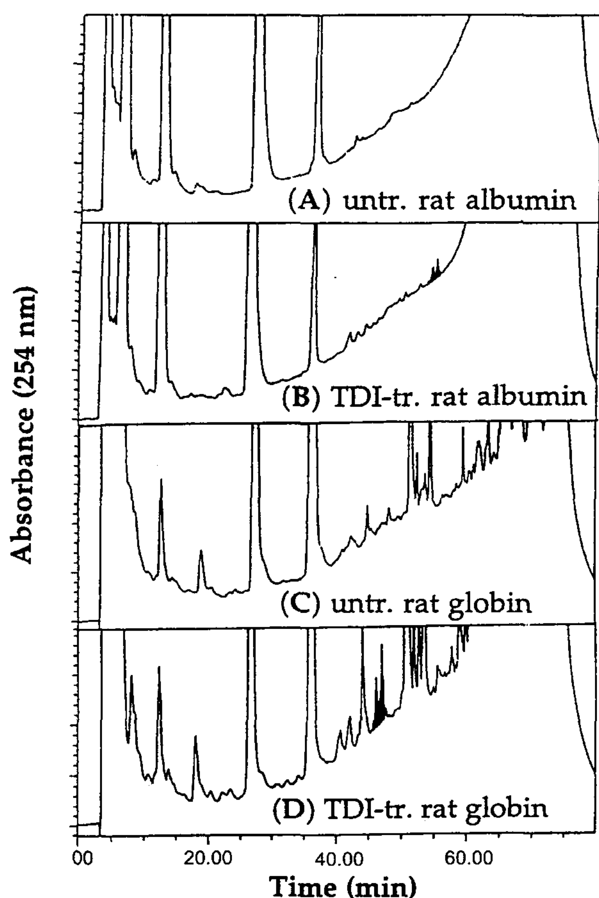

Fig. 4. HPLC chromatograms of albumin and globin of rats injected with single i.p. dose of TDI after pronase E digestion. Albumin isolated from TDI-treated rats showed adducts at 53 and 54 min (shaded peaks) as observed in vitro experiment while control rat albumin (A) did not. TDI-treated rat globin also showed adducts peaks observed *in vitro* at 46 and 47 min (shaded peaks), which were not detected in control globin (C).

manner (Table 2). The level of albumin adducts was approximately 10~20 times higher than in globin

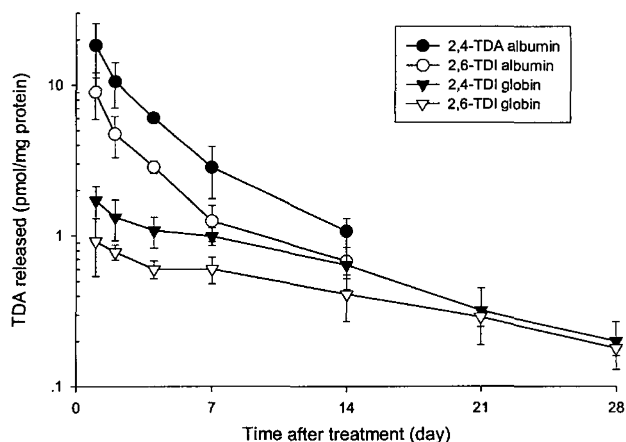

Fig. 5. Loss of TDI-derived protein adducts in rat blood as a function of time. A single group of rats was administered technical grade TDI (40 mg/kg) i.p. in hexane. At

at the same dose.

The nature of protein adducts was characterized by HPLC after pronase E digestion of albumin and globin (Fig. 4). Major adduct peaks in the HPLC chromatograms were eluted at 53 and 54 min for albumin and peaks at 46 and 47 min for globin, which are identical to those identified in *in vitro* experiment.

The kinetics of removal of adducts in blood proteins were considered (Fig. 5). The removal of albumin adducts proceeded with an initial $t_{1/2}$ of 1.2 day and slow phase occurred. In case of globin adducts, the removal of TDI adducts proceeded at much slower rate with a $t_{1/2}$ of 12.5 day. There was no obvious difference in the decay curves between 2,4- and 2,6-TDI.

4. Hepatic Protein and DNA Adduct

TDA released from hepatic DNA and proteins was monitored in order to evaluate the hypothesis that TDI is stable enough to attack directly liver tissue or TDA would covalently interact with hepatic DNA after metabolic activation. 2,4-TDI-DNA binding was detected at the level of 5.14±2.73 nmol/m DNA 1 day after the injection of 40 mg/kg TDI and rapidly decreased thereafter (Table 3). 2,6-TDI-DNA binding was not detected in our experimental system. Previous report indicated that'2,6-TDA did not result in hepatic DNA binding (La and Frolines, 1993). The levels of binding of TDI to hepatic

Table 3. Time course of the levels TDI adducts in hepatic DNA and proteins following administration of TDI to rats[a]

Time (day)	2,4-TDI adducts (pmol/mg protein or DNA)		2,6-TDI adducts (pmol/mg protein or DNA)	
	protein	DNA	protein	DNA
1	13.14±7.08[b]	5.14±2.73	3.14±1.66	nd[c]
2	12.76±4.25	1.88±1.10	2.48±1.56	nd
4	13.07±8.63	nd	2.22±0.69	nd
7	11.02±5.60	nd	2.35±1.48	nd

[a]Technical grade TDI was injected i.p. in rats at a single dose of 40 mg/kg body wt. Liver tissues were taken each indicated time after treatment with TDI and corresponding TDA was determined as described under Materials and Methods.
[b]Each value represents the mean±SD of determinations made with three rats.

protein 1 day after treatment were 3.4±1.6 for 2,6-TDI and 12.4±5.6 for 2,4-TDI. The levels of 2,4-TDI adduct were 4~6 times higher than those of 2,6-TDI adduct.

IV. DISCUSSION

The nucleophilic residues of protein or DNA are considered to react with the isocyanto groups of TDI (Fig. 6). Due to the unstable nature of the isocyanate functionality, spontaneous hydrolysis of TDI under aqueous conditions to give the corresponding amine species is also possible (Bartels *et al.*, 1993). Crosslinking seems to be derived from the reaction of both isocyanato groups with the target molecules prior to aqueous hydrolysis. Presuming direct reaction of TDI with the biological nucleophiles as the major pathway, the degree of binding or crosslinking depends on the stability of the isocyanato group of TDI in the body. Therefore, a considerable amount of DNA and protein adducts is believed to be formed by TDI and the majority of them is believed to be simple amide type as shown in Fig. 6. It has been suggested that TDI is not

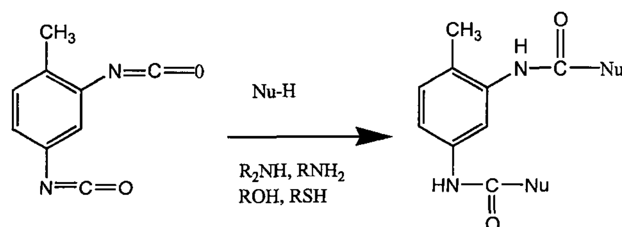

Fig. 6. Postulated scheme of interaction of TDI with macromolecules.

readily hydrolyzed in the biological system due to its high lipophilicity and the hemoglobin adducts of TDI observed in guinea pigs have proved this fact (Holden *et al.*, 1984; Day *et al.*, 1996). Degradation or repair of these adducts would release TDA, a known carcinogen, which may cause additional toxicity.

Incubation of TDI with calf thymus DNA under physiological condition demonstrated that TDI causes heavy crosslinking of DNA *in vitro*, as determined by the resistance to both enzymatic and thermal hydrolysis. Thermal hydrolysis (65~70°C) of DNA releases primarily purine bases and some of pyrimidine bases at higher temperature (>90°C). Based on the observed degree of base release from thermal hydrolysis of TDI-treated DNA, it is considered that TDI reacts nonselectively with the nucleophilic residues in both purine and pyrimidine bases. As a result of this, the structure of DNA is presumed to be changed so that the enzymes cannot approach to cleave the backbone. The bound TDI molecules were released under acidic conditions as a form of TDA, which was detected by GC-MS analysis. In the HPLC chromatogram, no base modification was observed after acidic hydrolysis, which indicates the bases of DNA and TDI molecules were held by amide bonds.

Animal experiment clearly demonstrated the binding of TDI to DNA as expected from the *in vitro* results. Hepatic DNA isolated from rats treated with single i.p. injection of TDI was identified to release TDA in the GC-MS analysis after acidic hydrolysis. Covalent binding of TDI to DNA in early period after TDI administration suggest that TDI can be transported to the liver or possibly to other organs without complete hydrolysis of the isocyanate moieties. Hepatic TDI-DNA adduct may be produced by direct incorporation with the formation of amide linkage. Comparing with *in vitro* result, however, DNA adducts are not so much produced at the low level of initial TDI influx because of higher competition ability of protein and weak reactivity of DNA. Thus, a portion of TDI binding to DNA in vivo might be generated by TDA, that was released from primarily TDI-macromolecular adducts and form covalent binding after metabolic activation in hepatocytes.

Protein adducts of TDI are considered to be as effective as DNA adducts in terms of long-term toxicity, regarding the lifetime and abundance of blood proteins with no repair system. *In vitro* experiments using untreated rat albumin and globin resulted in dose dependent covalent binding of TDI molecule to the protein and HPLC analysis showed several adducts after pronase E digestion. The adducted proteins released TDA when hydrolyzed under acidic conditions indicating the adducts are amide type as the case of DNA. The principal adducts observed *in vitro* were also found in albumin or globin of TDI-treated rats, which implies a considerable amount of TDI binds directly to the protein without metabolic activation. Characterization of the adducts as well as the site of modification on the protein deserves further investigation.

The levels of adduct in albumin by TDI administration were 8- to 14-fold higher than those in globin whereas the difference in *in vitro* binding was 2- to 8 times. There would be several reasons for the quantitative differences in binding level. Hemoglobin is segregated by the erythrocyte membrane and, thus, binding of TDI to globin requires the diffusion of TDI. In contrast, albumin may be alkylated both within the circulation and liver.

TDI-hepatic protein adducts were also detected after the treatment of TDI. Hepatic adducts were persistent up to 2 weeks after the treatment whereas albumin and globin adducts were removed in exponential mode. These results suggest that additional TDA or its surrogates were induced into liver after early hepatic protein adduct formation by direct TDI-reaction. Binding of TDI to protein or crosslinking may give rise to toxic effects and additionally, these adducted proteins have a potential to release a carcinogenic compound, TDA, constantly in the body by repair or degradation. Released TDA can be introduced to liver and form covalent binding with hepatic proteins after metabolic activation, as demonstrated by Patrick *et al.* (1996).

Taken together, TDI exposure is believed to exert long-term toxicity through its reaction with biological nucleophiles such as the bases of DNA and protein. We have provided substantial amounts of evidence associated with such type of binding to DNA both *in vitro* and *in vivo*. The chemistry involved in this type of intoxication is simple yet important, which is the formation of amide bond between the bases and TDI. The possibility of carcinogenesis by TDI has been taken into consideration due to the formation of TDA upon hydrolysis. Based on the results of this study, the carcinogenic potential of TDI is believed to not only exist but also persist by release of TDA from the adducted macromolecules in the body.

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