2,4-Toluene Diamines—Their Carcinogenicity, Biodegradation, Analytical Techniques and an Approach towards Development of Biosensors

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2,4-Toluene diamine (TDA), a class A carcinogen, is a major raw material for the production of toluene diisocyanate (TDI), which is one of the precursors for the production of polyurethane foams (PU). This review deals with 2,4-toluene diamine's (TDA) carcinogenicity, analytical techniques, biodegradation and use as a biosensor for biogenic and synthetic amines, emphasizing various carcinogenicity studies by 2,4-TDA on animals and humans. This review reports some publications of the analysis of body fluid samples of workers from a PU producing factory for presence of TDA and TDI, since TDI gets absorbed into the worker's body, getting metabolized into TDA. Biodegradations of 2,4-TDA by various researchers are reported and also our own research experience with biodegradation of 2,4-TDA using *Aspergillus nidulans* isolated from soil site at a polyurethane foam dumping site have been discussed in this review. Biosensors for various biogenic and synthetic amines are discussed.

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1 Introduction

2,4-Toluene diamine (TDA) is a major raw material for the production of toluene diisocyanate (TDI), which is used in the production of polyurethane (PU) foam. Both TDI and TDA have been subject to numerous environmental studies, many of which were sponsored by International Isocyanate Institute (III).¹ Commercial mixtures of 2,3- and 3,4- isomers, and 2,4- and 2,6-isomers are used as co-reactants, or as raw materials in the manufacture of urethane products, dye corrosion inhibitors, and rubber antioxidants. TDA isomers have relatively limited uses as epoxy curing agents and as photographic developers.^{2,3} Major sources of pollutants into the environment are from TDA manufacturing industries and their products. TDA is large volume intermediates used in the production of a wide variety of industrial and consumer products. Despite the wide range of

applications, there is a lack of information concerning their levels in the environment, as well as data on their transport and their fate in the ecosystem.² The isomers of 2,4- and 2,6-TDA are used predominantly as intermediates in the manufacture of toluene diisocyanate.

Aromatic amine compounds are among the environmental pollutants that are mutagenic in bacteria and mammals cells, and have been reported to be carcinogenic in rodents. Most of the aromatic amines are metabolized essentially through two steps: first *N*-oxidation by cytochrome P-450 enzymes^{4,5} and then *N,O*-acetyltransferase.^{6,7} The latter *O*-acetylation reaction occurs widely in bacteria and mammalian cells. The enzyme plays a pivotal role as an integral component for the phase II biotransformation process and it catalyzes several acetyltransfer reactions, such as arylamine *N*-acetylation, *N*-hydroxylamine *O*-acetylation and *N,O*-acetylation.⁸ Cunningham and Matthews⁹ used transgenic (Big Blue) mice to detect *in vivo* mutagenesis induced by TDA isomers. Their studies showed that 2,TDA leads to an increase in *in vivo* mutation frequency, whereas administration of the noncarcinogenic isomer 2,6-TDA failed to do so.

There is another source that contributes to TDA in humans. Polyurethane foams used with the Meme prosthesis are not

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appropriate for the application of breast implant. It has been reported that foam can be hydrolyzed *in vitro* by (3 N) sodium hydroxide at 150°C and that this hydrolysis leads to the formation of toluene diamine isomers. Clinical observations proved that implanted polyurethane foams are neither inert nor permanent as they slowly disintegrated and were absorbed in tissue. This process takes several years to complete and the kinetics and histology are still unclear. 2,4- and 2,6-TDAs have been found in the urine of patients after polyurethane foam-coated silicone breast implantation.¹⁰ Studies on the identification of TDAs in the urine would be clinically significant in order to investigate how the foam breaks down in the human body and how the breakdown products are stored or excreted.¹¹

2,4-TDA could be analyzed using techniques like TLC, GC, HPLC, GC HPLC, MS coupled with GC and HPLC according to the nature and concentration of sample. Biosensor research shows promising possibilities for analyzing the carcinogenic compounds on-line during industrial production and monitoring in the blood, urine and tissues of workers in PU-producing industry and in the environment. Authors share their own experience with biodegradation of 2,4-TDA using *Aspergillus nidulans* isolated from a soil site where polyurethane had been dumped.¹²

2 Carcinogenicity of 2,4-Toluene Diamine

2.1 TDA and their isomers

The majority of chemicals that display carcinogenicity cause DNA damage such damage, if unrepaired, gives rise to mutations, and may lead to tumor development through complex, poorly understood mechanisms. In most cases, the chemical is unable to interact directly with DNA, but may readily interact through metabolically formed reactive intermediates generated within the living organism.¹³ The four possible isomers differ in their carcinogenic potential. 2,4-TDA has been described as a moderate carcinogen, inducing tumors in the livers of both rats and mice.¹⁴⁻¹⁶ The 2,5-isomer was tested for carcinogenicity only as a component of hair dyes, which also included the carcinogenic 2,4-TDA, and, consequently, its carcinogenic potential cannot be independently evaluated. In the Ames test, in the presence of an activation system, it provoked a positive mutagenic response.¹⁷ 2,6-TDA is classified as a noncarcinogen, having failed to induce tumors in both rats and mice,¹⁶ but in the Ames test, the presence of an activation system, it elicits a mutagenic response.¹⁷

Cheung *et al.*¹³ reported that 2,4-TDA is readily *N*-hydroxylated by hepatic microsomes. The resulting hydroxylamine is genotoxic, as evidenced by the positive response in the Ames test; on repeated administration it self-induces its bio-activation and it interacts with Ah receptor, thus causing cellular proliferation and promotion of mutated cells. Poor rate of *N*-hydroxylation of 2,4-TDA indicates that it is an ultimate carcinogen. However, 2,6-TDA does not induce CYP1A activity and its own activation. In addition, it does not interact with Ah receptor and thus it is unlikely to function as a tissue proliferator. Indeed, the isomer, in contrast to 2,4-TDA, failed to release hepatic cell turnover when administered to rats.¹⁸ These observations may explain why 2,6-TDA is a mutagen but not a full carcinogen. An additional consideration is that it may be rapidly deactivated through ring hydroxylations and *N*-acetylation. It is, however, pertinent to point out that the metabolites of 2,6-diaminotoluene where both amino groups

Table 1 Carcinogenicity of diamines

Source/type of test done	Effect	Ref.
Rats and mice	Moderate carcinogen, tumor inducers	14 – 16
Ames test, rats and mice	Mutagenic response	17
Ames test, rats and mice	Self induces its bio-activation and interacts with Ah receptor	13
Rats	Mutagenic activity	19

had undergone acetylation retained their mutagenic activity.¹⁹ 2,3-TDA has minimal genotoxic potential, possibly reflecting its poor *N*-hydroxylation, it does not induce its own activation, and consequently it is unlikely to be carcinogen, per second. However, it induces CYP1A activity and may act as a cocarcinogen by potentiating the effect of carcinogens that rely on this subfamily for their activation. It also binds to the Ah receptor and so may function as a promoter of mutated cells. 2,5-TDA is a weak mutagen despite its high rate of *N*-hydroxylation, so it has been predicted that 2,5-TDA is unlikely to be a carcinogen. It is hence concluded that *in vitro* studies need to be based on the understanding of the pathways of activation, of the enzymes catalyzing these genotoxic potentials of a given chemical. This approach ensures that long-term and highly expensive carcinogenicity studies are not conducted needlessly.¹³

2.2 Studies of toxicity on humans

Quantification of isocyanates and amines in trace levels in air has been of great interest for many years due to their possible environmental and occupational hazards. TDA has also been detected in urine hydrolysate after occupational exposure to TDI.²⁰ Diisocyanates are highly reactive with many compounds containing active hydrogen, for example, carboxyl and amino groups in proteins. It is therefore likely that TDI reaches the tissue initially, rather than being absorbed and distributed throughout the body as an unchanged compound. Persson *et al.*²¹ analyzed urine and blood samples of PU foam producing factory workers on six occasions for eight studied subjects during a two day period. Two workers had an elimination rate of 20 – 70 ng per hour on average for 2,6-TDA and 2,4-TDA per hour and three PU workers had an average of 100/300 ng TDA. The elimination rate curves for all studied subjects had a linear relation with exposure to TDI, whereas 2,4-TDA and 2,6-TDA concentrations in plasma for PU factory employees were virtually stable. No relation between the elimination rates of TDA in urine and the plasma concentrations of TDA was found. The five workers showed plasma concentrations of the sum of 2,4-TDA and 2,6-TDA in the range of 1 – 8 ng per TDA per ml of plasma. Skarping *et al.*²² analyzed amine and isocyanates in air and biological samples, such as urine and blood, that have been the subject of increasing interest in recent years owing to their environmental and occupational hazards. Lorenzi *et al.*¹¹ studied PU foams, which are extensively used as cover-textured breast implants. It is important to know if this material can cause secondary cancers including breast cancer. There are reports in the literature associating PU foam-covered implants with tumor formation in rodent models. TDI is readily hydrolyzed to 2,4- and 2,6-TDAs, which have been shown to be mutagenic and to produce liver cancer in rats. Lind *et al.*²³ analyzed the blood and urine of eleven workers at two flexible foam PU production plants, and concluded that the half life in

Fig. 1 Schematic representation of the chemical transformation of TDI in water into TDA.

plasma of chronically exposed workers for 2,4- and 2,6-TDA was twice as long as that for volunteers with short-term exposure. An indication of a two-phase elimination pattern in urine was found. The first phase was related to the more recent exposure; the second, much slower one was probably related to release of TDA in urine from TDI adducts in the body. Lee and Phoon²⁴ have studied diurnal variation in peak expiratory flow rate (PEFR). This was studied in 26 mixers from eight factories making polyurethane foam, who were exposed to TDI, and in 26 unexposed controls matched for age, race, and smoking habits all were men. In this monitoring, authors reported that one of 24 environmental samples taken exceeded the short-term exposure limit of 0.02 ppm for TDI. They conclude that foam workers may still have high exposure to TDI, high prevalence of irritative symptoms, increased diurnal variation in PEFR and evidence of chronic airway obstruction, particularly those with ≥ 10 years of exposure.

3 Permissible Levels of TDA

The World Health Organization² reported that two properties of TDA are relevant to this problem. Since vapor pressure is low (0.34×10^{-3} mm Hg at 37.8°C) the risk of contaminating the environment through evaporation is minimal. Solubility of 2,4-TDA in 7.74 g/L, Hazardous Substance Data Bank²⁵ reports that the potential for exposure through water contamination is of vital concern. No data are available on levels of TDA in air and water, since most TDA produced and is used on-site by the manufacturer; therefore published production figures do not adequately reflect the true world production of TDA.

4 Biodegradation of 2,4-TDA

Detailed data are lacking on the extent use of TDA, their transport, distribution, and degradation within the environment, though a few pieces of information are available.² Bench-scale treatment studies for the 2,4-TDA using acclimated sludge from a treatment plant showed that the isomers of TDA are degradable. The observed total organic carbon removal was 45% in 4 h.²⁶ The Japanese Ministry of International Trade and Industry (MITI) test indicated that TDA was not degradable.²⁷ On the other hand, it has been reported that TDA could be treated biologically through precise conditions of biodegradation.²⁷ Asakura and Okazaki¹ have successfully metabolized the 2,4-TDA in sludge using the microorganic enzyme system by acclimating the sludge for about 200 days with aniline and TDA. In this study, a microorganic enzyme system, which metabolized TDA, was obtained by acclimating the activated sludge with aniline and TDA. In the sludge subject to 200 days acclimation, a considerable increase in respiration rate with the addition of TDA accompanied the

Table 2 Analytical techniques for 2,4-TDA

Source	Biodegradation/removal	Ref.
Sludge	Microorganic enzyme system	1
TDA lab-scale (not isolated from anywhere)	Anaerobic degradation	28
TDA lab-scale (not isolated from anywhere)	Mixed culture	12
TDA lab-scale (not isolated from anywhere)	<i>Aspergillus nidulans</i>	12
TDA lab-scale (not isolated from anywhere)	Response surface methodology for media optimization	12

sharp decrease in its concentration. This indicated that TDA was metabolized fortuitously. The rate of biodegradation of TDA in the absence of aniline was first-order with respect to its concentration, when the initial TDA concentration was less than about 5 mg/L. From these studies, the authors concluded that metabolism of TDA can be classified as a fortuitous. This indicates that the co-existence of carbon and energy source such as aniline is not necessary for the biodegradation of TDA. The biological treatment of TDA seems to be very practical if one applies the acclimation processes to activated sludge. Asakura and Okazaki¹ reported that they are also carrying out pilot plant scale studies in their laboratory based on the above research findings. Freedman *et al.*²⁸ carried out the anaerobic degradation of TDA with ethanol and ether as cosubstrate. No decrease in TDA was observed in the first 77 days, but from 77 to 99 days TDA was readily consumed as sole substrate every 2 to 4 days, and consequently there was very low COD in the effluent due to oxidation of TDA. Much commercial, general-purpose foams are still synthesized from diol-terminated polyesters and aromatic diisocyanates.²⁹ TDI is sensitive to water and to active-hydrogen containing compounds. As a result, some of it reverts to TDA in water (refer to Fig. 1). This gave us some very strong evidence that PU have unreacted TDI. An additional point of support was that organisms that could survive for many years in the dumped solid PU could have assimilated TDA and such impurities as sole carbon source. This prompted us to isolate the microorganisms from where PU had been dumped soil. Forty fungi were isolated from PU such soil sites and, based on initial degradation trials, *Aspergillus nidulans* was shortlisted. Parameters like pH, glucose concentration, time of 2,4-TDA addition in the medium were optimized using Response Surface Methodology (RSM). We believe that this is probably the first attempt to degrade 2,4-TDA using single strain,¹² whereas previous researchers have carried their project out using mixed cultures. Initial results are very encouraging and exciting (results not reported in this manuscript).

5 Analytical Techniques for 2,4-TDA

Selection of the analytical techniques for quantifying TDA mainly depends on the nature of the sample; biological fluids, hair, consumer products air and water. It could also be helpful to choose a specific technique depending on the concentration of 2,4-TDA and the nature of sample. For selected analytical techniques for TDA, refer to Table 3.

There have been reports³⁰ that the 2,4-, 2,5-, 2,6- and 3,4-isomers in HPLC could be found with UV electrochemical

Table 3 Analytical techniques for 2,4-TDA

Method	Column specification	Mobile phase	Ref.
GC	GC-GSP-20 M column (Perkin-Elmer, Norfolk, CT)	Injector and detector temperatures were set at 250°C. The chromatographic oven was programmed to raise the column temperature from 50°C to 100°C at 30°C/min followed 5°C/min up to 240°C.	29
GC/MS	GC-MS A Hewlett-Packard HP-Model HP-5890 and HP-5970 MS using a split port injector with a DB-5 column		
GC-MS	VG-Quattro MS (Fisons Instruments, UK) connected with GC with an A 200S autosampler (Fisons Instruments, Italy)	Hot needle technique for the syringe. Starting temperature was 110°C for 1 min, then raised at 15°C/min to 280°C and kept for 2 min.	
		GC-MS interface temperature was 280°C, the capillary inlet pressure of helium was 0.8 kg/m ² .	22
HPLC	TSK-gel ODS-TM (Tosoh Co., Japan) with UV detector (Shimadzu Co., Japan)	Acetonitrile-water (40 +60) contained 16 mmol/l KH ₂ PO ₄ + 4 mmol/l K ₂ HPO ₄ as an eluent at flow rate of 1 ml/min.	1
GC-FID	Hewlett-Packard 5730 A GC with ion flame ionization detector	Glass column packed with 60 – 80 mesh Tenax GC porous polymer at 240°C isothermal temperature.	47
HPLC	C-18 column (Vydac) in conjunction with Module 1 system. Detection was by UV 254 nm.	Isocratic analysis of TDA 39.5%, 59.5% and 1% triethylamine at flow rate of 1.3 ml/min.	28
LC	Hewlett-Packard 1090L liquid chromatograph with diode-array and fluorescence detection	LC separations with 25 cm × 4.6-mm i.d. Vydac 201TP54 polymeric C-18 column with 5 µm particles size.	48

detection in the range of 0.2 – 0.7 ng in water; the determination of 2,4-TDA had a detection limit of 3 µg/m³ in air.³¹ TDA has also been analyzed in air by means of HPLC.³² There are research findings³³ of the determination of 2,4- and 2,6-TDA with electron detection on GC column and the determination of 2,4- and 2,6-TDA isomers with GC with nitrogen-phosphorous detector on glass capillary columns with a detection limit of 10 – 20 pg amine.³⁴ 2,4- 2,5- and 3,4-Isomers were determined in hair dyes.³⁵ The use of HPLC in conjunction with ultraviolet detection for determination of 2,4-, 2,5-, and 2,6-isomers with sensitivities of 0.5 mg/L in hair dyes has been reported.³⁶ There have been reports that, 2,4- and 2,6-isomers of TDA determined using HPLC with UV and GLC-MS with sensitivities to the range of 0.1 µg/L,^{37–39} and, using TLC, exactly 1 µg/g of concentrations 2,4- and 2,6-TDA were determined.⁴⁰ In biological tissues and fluids 2,4- and 2,6-TDA were analyzed using HPLC with UV.⁴¹ In isomeric mixtures, all isomers of TDA were analyzed using NMR,⁴² TLC,⁴³ GC with flame ionization detector,⁴⁴ GC with thermal detector⁴⁵ and 2,4- and 2,6-TDA were analyzed using IR.⁴⁶

6 Biosensor for Diamines

There have been descriptions based on new concept for electrochemical biosensing of DNA sequences, and for using electrical fields to regulate DNA interactions in connection with the development of genoelectronic chips. Progresses have been made on a new electrochemical strategy for trace measurements of toxic aromatic amine compounds.^{49,50} This device relies on the intercalative collection of aromatic amines onto the immobilized dsDNA layer, followed by potentiometric stripping

quantitation of the accumulated species. Having the amino substituent in a slightly different position produces a dramatic effect upon the response. Nanomolar detection limits are obtained after a 10-min accumulation. Detection limits around 1.8×10^{-6} mol/l 1,2-diaminoanthraquinone, 2×10^{-8} mol/l 2-anthramine, 1×10^{-8} mol/l 1,2-diaminoanthraquinone, 2×10^{-8} mol/l 9,10-diaminophenanthrene, and 6×10^{-8} mol/l 1-aminopyrene can be estimated from signal to noise characteristics of these data ($S/N = 3$). The above detection limits are low enough for assays of polluted environmental samples. Electrochemical biosensors resulted in a rapid, sensitive, and simple detection of aromatic compounds.

Recent reports discuss an electrode for biosensors by covalent immobilization of the redox enzyme (Horse Radish Peroxidase).⁵¹ The first step was heating the matrix in a covalently modified electrode at 100–110°C to remove volatiles and absorbates. The second step was chemical pretreatment to introduce functional groups like –OH, –NO₂ and –Br. The third step was glutaraldehyde coupling of the enzyme using carbodiimide. HRP enzyme was tested in a rotating disk experiment to determine its response with the substrate. This covalently-modified electrode has potential applications for biosensors for various compounds.

Previous researchers⁵² have immobilized amine oxidase from grass pea (*Lathyrus sativus*) seedlings and fungus *Aspergillus niger* and these immobilized oxidase used to construct flow enzyme reactors for amine assay with spectrophotometric detection of enzymatically produced hydrogen peroxide. The optimized biosensor, with an average lifetime of 20 days, showed a linear response to the amount of histamine in the range 7.0 – 90 nmol, to the amount of with assay limit of 4.4 nmol and putrescine in the range of 0.9 – 70 nmol, with the

Table 4 Biosensors for diamines

Type of sensor	Range of detection	Ref.
Immobilization of dsDNA, potentiometric stripping quantitation	Nanomolar range	49,50
Covelent immobilization-HRP	Nanomolar range	51
Immobilization of amine oxidase (<i>Lathyrus sativus</i>)	Nanomolar range	52
Pea seedlings amine oxidase	Nanomolar range	53
Electrochemical detection, immobilization of diamine oxidase	Detection up to 6 $\mu\text{mol/l}$	54
Cross-linked putrescine oxidase	Detection range (0.5 – 300 $\mu\text{mol/l}$)	56
Amine oxidase	—	57

assay limit of 0.5 nmol. Another group⁵³ prepared a new inorganic based sorbent on modified triazine {2-[(4,6-bis aminoethylamine)-1,3,5-triazine]-silasorb} which binds pea seedlings amine oxidase (PSAO) very tightly, without loss of its catalytic activity. This unique feature was used for the basic characterization of 55 biogenic and synthetic amines. The result is very interesting indeed. It showed high sensitivity to putrescine $20.0 \pm 0.64 \text{ mA mol}^{-1}$ ($636.9 \pm 2.03 \text{ mA mol}^{-1} \text{ cm}^{-2}$), a limit of detection of 10 nmol (determined with respect to a signal-to-noise ratio 3:1), a linear range of current response to 0.01 – 100 μmol amount of substrate and good reproducibility. It was concluded that this sensor could be used to future industrial and clinical analyses.

Recently another research group⁵⁴ determined biogenic amines with an electrochemical biosensor. The immobilization of diamine oxides (DAO) on a nylon-net membrane, using glutaraldehyde as cross-linking agent; phosphate buffer at pH 8.0 was used. The detection limit was $5 \times 10^{-7} \text{ mol/l}$. The linear range common to the amines tested was observed from 1×10^{-6} to $5 \times 10^{-5} \text{ mol/l}$ and it was concluded that changes in the concentration of biogenic amine content in salted anchovy samples could be measured with this biosensor and IC-IPAD methods. The same trend was observed in fish during storage. Bouvrette *et al.*⁵⁵ developed an amperometric biosensor for diamines using a diamine oxidase purified from porcine kidney. This biosensor using immobilized diamine oxides (DAO) and platinum electrode (poised at +700 $\mu\text{mol/l}$ vs. Ag/AgCl for determination peroxide released from the enzymatic oxidation) was linear up to 6 $\mu\text{mol/l}$ histamine, cadaverine, or putrescine with a lower detection limit of 25 $\mu\text{mol/l}$. An amperometric diamine sensor for clinical applications in diagnosis of bacterial vaginosis has been reported.⁵⁶ This sensor is based on crosslinked putrescine oxidase (PUO), which catalyzes the conversion of diamines (mainly putrescine and cadaverine) to products including hydrogen peroxide. This sensor has a linear dynamic range from 0.5 – 300 $\mu\text{mol/l}$ for putrescine that covers the expected biological levels of the analyte. Studies on the influence of the main factors of biosensor selectivity on monoamine determination have yielded very interesting results.⁵⁷ In the composition of new biosensors, amine oxidases (AO) from different sources were used. Mitochondria AO from pig and rat liver, and AO from *Methanosarcina barkeri*. New methods for the individual determination of monoamines and for the detection of their sum in tile samples have been worked out.

7 Conclusion

The basic aim of this review is to come out with the idea that there are different possibilities of development of sensors for diamines. Hence, the aspects like permissible level, analytical techniques, biodegradation and biosensors were discussed, with special emphasis on TDA. This review would give broad ideas for researchers who are working on amines and related compounds. From our own experience with biodegradation and development of microbial biosensors for various compounds,⁵⁸⁻⁶¹ we conclude that, even for complex chemicals like TDA, it is worth the attempt to try developing microbial sensors. Isolation of microbes from the substrate-contaminated soil would open up new opportunities of research. By studying isolated strain(s) biochemical characterization, we may come across unique properties of isolated strain and it may lead to microbial biosensor for various chemical compounds. The improvements in molecular biology give us now immense opportunities for improvement of a potential strain for various applications.

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