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Genetic Toxicology and Environmental Mutagenesis

Chemical behaviour of seven aromatic diisocyanates (toluenediisocyanates and diphenylmethanediisocyanates) under in vitro conditions in relationship to their results in the Salmonella/microsome test

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Abstract

There are conflicting results on the mutagenicity of toluenediisocyanate (TDI) and diphenylmethanediisocyanate (MDI). It was found that the organic solvent chosen to dissolve the compounds dictates the outcome of the bacterial tests. The Salmonella/microsome tests showed uniformly mutagenic effects for all the compounds that were predissolved in DMSO. Due to the instability of aromatic diisocyanates in DMSO this solvent was replaced by ethyleneglycoldimethylether (EGDE). TDI and MDI endured the dissolving and were therefore still available for the subsequent bacterial tests. Furthermore, no aromatic diamines (TDA or MDA) could be detected in EGDE prior to the start of the assays. The Salmonella/microsome tests, however, revealed unexpected differences between TDI and MDI. As previously published the four types of MDI showed negative results, whereas the data presented in this paper demonstrated mutagenic effects of all three types of TDI if EGDE is the solvent. To gain deeper insight into the chemical changes that occurred during the Salmonella/microsome test, the possible reactions were modelled in the laboratory by mixing predissolved diisocyanates with a defined surplus of water and monitoring the progress of the chemical reactions by analytical methods. Additionally, the quality of the model was checked by exposing solutions of 2,6-TDI and 4,4′-MDI to the real biological test environment. In both cases, the reaction patterns of TDI were different to those of MDI. Within 1 min, which is the maximum time needed to mix the predissolved compounds with water before they are poured onto the agar plate, the TDI content was reduced in favour of different ureas and TDA. In addition water was replaced by the complete set of test ingredients. While the TDA content remained more or less constant, the amount of residual TDI was reduced considerably. Reactions of MDI were markedly slower than those of TDI. More than 90% of the predissolved MDI remained intact when it was mixed with water. The biological test ingredients accelerated the reduction of the MDI content. Within 45 s, more than two thirds of the MDI disappeared. Evidently, the chemical reactions continue during incubation. It is assumed that the contrasting results of TDI and MDI in the Salmonella/microsome test are due to the different reaction patterns—and reaction products—of the predissolved diisocyanates created under the specific conditions of the test. These findings indicate that the chemical interactions between

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reactive test compounds and solvents or test media need to be considered in the interpretation of the relevance of test results.

Keywords: Salmonella/microsome test; Toluenediisocyanate; Diphenylmethanediisocyanate; Dimethylsulfoxide; Ethyleneglycoldimethylether; Chemical reactions during bacterial tests; Hydrolysis; IR spectroscopy; HPLC

1. Introduction

Aromatic diisocyanates like toluenediisocyanate (TDI) and diphenylmethanediisocyanate (MDI) find widespread use in the manufacture of flexible polyurethane foams for bedding, furniture, cushions, carpet underlay and in transportation seating. They are also used in rigid foams for insulation, shoe sole systems, elastomers, coatings, adhesives, sealing compounds and a variety of miscellaneous applications [1,2]. They comprise a family of compounds which contain the reactive functional group N=C=O, the key to the chemistry of polyurethanes (PUR). TDI, with its most important product TDI 80, a 80:20 mixture of the 2,4- and 2,6-isomers, and MDI, including p-MDI, the 'polymeric' grade of this product (which usually contains 40–60% monomeric MDI), have by far the greatest importance in the marketplace. The global production capacity of each of these diisocyanates exceeds a million tons/year.

There are contradictory results about the mutagenicity of TDI and MDI in Salmonella typhimurium strains [3–8]. The conclusions drawn by some of the authors have been subject to critical discussions [9,10]. A survey of investigations on MDI is given in Ref. [11].

As in all biological and environmental assays, in vitro mutagenicity tests are conducted in aqueous media. Aromatic diisocyanates, which are practically insoluble in water and react only at the interface, are usually dissolved in an organic solvent—in which both the diisocyanate and water are soluble—prior to the use in the assay [12–16]. Dimethylsulfoxide (DMSO) is commonly the preferred solvent.

The choice of the solvent, as well as its possible water content, may be one of the reasons for the differing mutagenicity test results reported in the past. Aromatic diisocyanates are able to react with the moisture that is always present in 'dried' organic solvents and to give rise to a number of products, including ureas, oligoureas, polyureas, carbon dioxide, and possibly traces of aromatic amines, which are known to be mutagenic. This chemical conversion begins prior to treatment in the assay and may be catalysed by the solvent itself. Reactions of N=C=O groups with water can also be expected when the predissolved diisocyanates are mixed with the aqueous test ingredients and when this mixture is incubated on solid agar. Furthermore, the chemistry of aromatic diisocyanates suggests competing reactions of N=C=O groups with biological NH_2, NH, SH, OH or COOH groups on proteins or polysaccharides [2,17].

Adding predissolved diisocyanates to the Salmonella/microsome test environment — bacteria, nutrient broth, S9 mix, buffer, soft agar — basically means highly dispersing a small amount of the compound in a huge surplus of water. The interactions of water and aromatic diisocyanates are complex and may involve several mechanisms. It is a common misconception that in the presence of water, aromatic diisocyanates are converted to aromatic diamines in stoichiometric proportions. This is certainly not the case. However, an important question is to what extent and when—if at all—do aromatic diisocyanates that are dissolved in an organic solvent give rise to traces of aromatic diamines in a Salmonella/microsome test environment.

During our studies on the chemical fate of aromatic diisocyanates in the Salmonella/microsome test, we observed that neither 2,4-TDI nor 4,4′-MDI was stable in DMSO. In model solutions based on concentrations normally used in a Salmonella/microsome test, we noticed that traces of water that are always found in dried commercial DMSO (even if thoroughly distilled) degraded the diisocyanates and led to a number of reaction products, including small amounts of 2,4-diaminotoluene (2,4-TDA) or 4,4′-diaminodiphenylmethane (4,4′-MDA). We also observed that under these homogeneous conditions, reaction with water was fast and led to the complete disappearance of 2,4-TDI or 4,4′-MDI within minutes [18]. Unfortunately, information on the stability of diisocyanates in the chosen solvent is scarcely

given in literature. The real chemical compositions of the solvent/diisocyanate solutions prepared for the test are thus often uncertain. We therefore assume that most of the published Salmonella/microsome test results for TDI and MDI in reality reflect effects of DMSO-specific degradation products and not of the original diisocyanates.

This assumption agrees with the results of comparative studies carried out with four types of MDI using the polar, hygroscopic DMSO and the less polar etyhleneglycoldimethylether (EGDE) as solvents [19]. The choice of the organic solvent dictated the outcome of bacterial tests. Positive results were obtained for DMSO solutions of all four types of MDI, while uniformly negative results were found when the diisocyanates were dissolved in EGDE. These findings reflect the results of analytical investigations. Small amounts of MDA were discovered in DMSO, while no MDA could be detected in EGDE.

To gain deeper insight into the chemical changes that occur in the Salmonella/microsome test, we monitored the fate of seven aromatic diisocyanates in modelled and real test environments. The analytical results obtained were compared with the results of the bacterial tests.

2. Reactions and mechanisms in aqueous media

The reactions between phenylisocyanate—the simplest of the aromatic isocyanates—and water have been intensively studied by Naegeli et al. [20]. They concluded that the reaction could follow several pathways leading to symmetrical urea and/or, under certain conditions, to aniline, an aromatic amine (Fig. 1). Most of these reactions are reversible, with the exception of those that split off carbon dioxide or water. It was suggested that the rate-determining step is the formation of a carbamic acid. This unstable compound continues to react or decomposes via one or more of several intermediates to give rise to a urea. The adopted path is strongly influenced by the prevailing reaction conditions, especially if the system is homogeneous or heterogeneous. The course via the amine to the urea may be partially or completely interrupted at the stage of the amine. This

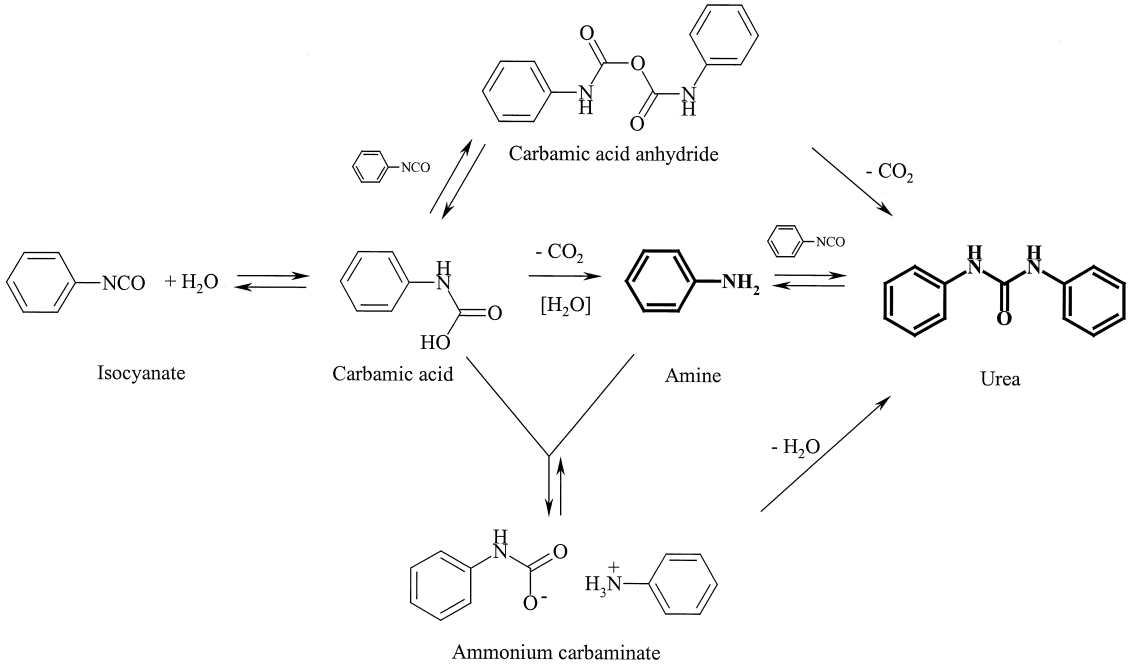

Fig. 1. Reactions of phenyl isocyanate and water.

interruption may occur in strong acidic (low pH levels lead to protonation of the amino groups) and in strong basic environments (formation of carbamic acid salts). This may also happen in cases where the amine is an insoluble precipitate that escapes further reaction, or where it possesses a low nucleophilic power due to steric hindrance and/or electron withdrawing groups.

In a recent study, semiempirical quantum mechanical calculations have been applied to detect the dominant reaction sequence in the uncatalyzed, homogeneous phenylisocyanate/water reaction (Fig. 1). The study indicated that the reaction proceeds not primarily by the decarboxylation of the carbamic acid to the amine, but rather along the carbamic acid anhydride path, which is four orders of magnitude faster [21].

Reactions of diisocyanates in aqueous media are even more complex than those of a monoisocyanate like phenylisocyanate. In Fig. 2, a sequence of possible reactions following the interaction of 2,4-TDI and water is shown. A similar sequence can be illustrated for MDI. Because of the difference in reactivity of the two N=C=O groups, the reaction of TDI with water is not simply hydrolysis to the aminoisocyanate (TDAI) or to the diamine (TDA). These are merely intermediates in a series of fast reactions which consume more N=C=O groups of other molecules and lead predominantly to ureas (TDI-U, TDAI-U, TDA-U), oligoureas and solid, insoluble polyureas. The composition of the final product mix is influenced to a great extent by the prevailing reaction conditions (heterogeneous or homogeneous, pH value, temperature, concentration, presence of catalysing substances, etc.) and also by the miscibility and solubility of the various compounds.

Heterogeneous reactions usually occur under environmentally relevant conditions, for example, when a diisocyanate comes into contact with water following an accidental spillage. TDI — or MDI — is relatively dense and hydrophobic. When dropped into water, it sinks and agglomerates. Reactions occur only at the interface of globules, and because of the difunctional nature of TDI, a polymerisation reaction starts, leading predominantly to polyureas via the stage of ureas and oligoureas. These form a crust encapsulating unreacted TDI. The polyureas produced within and beneath this crust are to some extend hydrophilic and support the diffusion of water into the globules. At the same time, they hinder diffusion of any diamine or other soluble species from the reaction site and hence promote the production of more polyurea and markedly reduce the overall yield of diamines. The globule of TDI thus gradually solidifies from the interface inward [22].

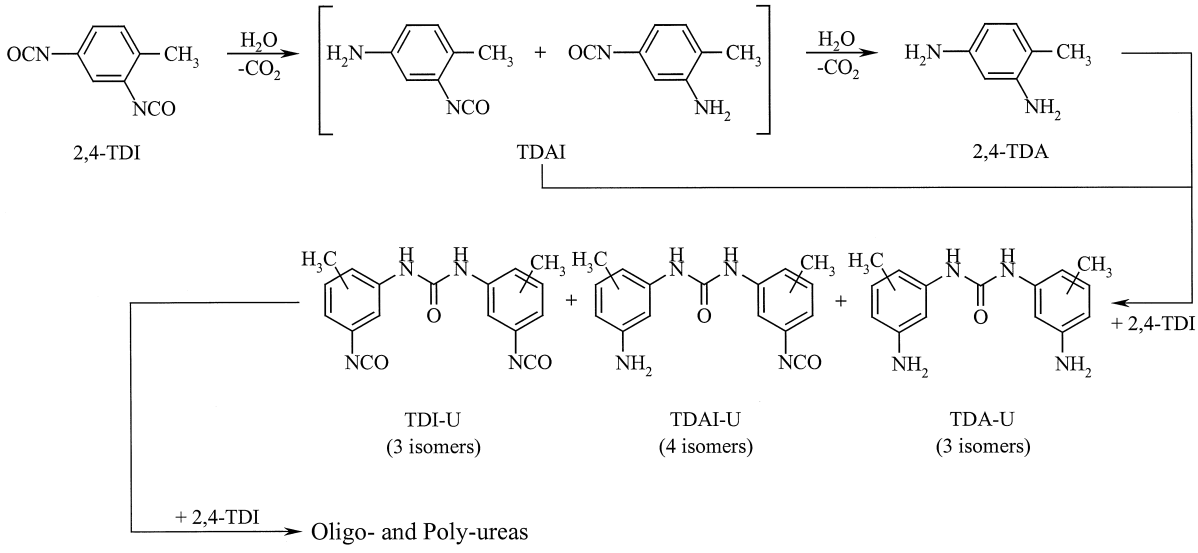

Fig. 2. Reactions of 2,4-TDI and water.

In the laboratory, the diisocyanate/water reaction may be conducted heterogeneously or homogeneously. The technique used for introduction and dispersal of the test chemical can significantly affect the physical form and chemical composition of the reaction products, which may be ureas, oligoureas, polyureas, amines or others [23–28].

In heterogeneous systems in which the diisocyanate and water are not mutually soluble, reactions are normally slow. In laboratory studies, the reactions are usually accelerated by allowing a controlled contact between TDI (or MDI) and water for a set period of time. This may be achieved by different rates of stirring or by dispersing predissolved diisocyanates in water. These artificial conditions, which cannot be compared to those in the natural environment, lead to a high degree of dispersion that prevents agglomeration and increases the surface area of the diisocyanate globules. Such conditions are generated in the Salmonella/microsome test when the predissolved TDI — or MDI — is mixed with the aqueous test ingredients.

In the course of homogeneous reactions, both the diisocyanate and water are soluble in the test medium. Such conditions are typically found when a diisocyanate is dissolved in a moist organic solvent, as it is the case prior to its use in a bacterial test. Polar, aprotic solvents like DMSO or dimethylformamide (DMF) additionally accelerate the reactions with water.

3. Materials and methods

3.1. Chemicals

All diisocyanates were obtained from Bayer, Germany.

- 2,4-diisocyanatotoluene (2,4-TDI), Desmodur® T 100, CAS-No. 584-84-9,
- 2,6-diisocyanatotoluene (2,6-TDI), CAS-No. 91-08-7,
- 80:20 mixture of 2,4- and 4,6-isomers of TDI (TDI 80), Desmodur® T 80, CAS-No. 26471-62-5,
- 4,4′-diisocyanatodiphenylmethane (4,4′-MDI), Desmodur® 44 M, CAS-No. 101-68-8,
- 2,4′-diisocyanatodiphenylmethane (2,4′-MDI), CAS-No. 5873-54-1,
- Mixture of isomers of monomeric MDI (4,4′-, 2,4′-, and 2,2′-MDI), CAS-No. 26447-40-5,
- Polymeric MDI, Desmodur® 44 V 20, CAS-No. 9016-87-9.

Reference substances were obtained from, or specially prepared by, the Polyurethane Research Dept. of Bayer.

Dibutylaminoureas of TDI and MDI,

2,4-diaminotoluene (2,4-TDA), CAS-No. 95-80-7,

2,6-diaminotoluene (2,6-TDA), CAS-No. 823-40-5,

4,4′-diaminodiphenylmethane (4,4′-MDA), CAS-No. 101-77-9,

2,4′-diaminodiphenylmethane (2,4′-MDA), CAS-No. 1208-52-2,

N,N′-bis-[3-isocyanato-4-methylphenyl]urea (4,4′-TDI-urea, TDI-U),

N,N′-bis-[3-amino-4-methylphenyl]urea (4,4′-TDA-urea, TDA-U), CAS-No. 101086-45-0.

Other chemicals were obtained from the following sources: EGDE (Merck), acetonitrile Chromasolv® and dibutylamine (Riedel-de Haen), DMSO for IR spectroscopy (Merck). DMSO samples had been dried and stored over molecular sieves but still contained 0.01–0.1 wt.%/wt.% of water.

3.2. IR spectroscopy

The respective diisocyanate was weighed into a volumetric flask and made up to 100 ml with solvent. Samples were removed from the solution at set intervals (during a 4 h period), transferred to a CaF_2 cuvette (0.099 mm) and an IR spectrum was taken (FT-IR spectrometer PE 1700, resolution 4 cm^{-1}, 10 scans). The concentration of the NCO band (2270 cm^{-1}) was quantified by means of the base line procedure, which uses the peak height for the calculation. Calibration was carried out in 1,2-dichlorobenzene (the diisocyanate made up to 100 ml with 1,2-dichlorobenzene, extinction measured at 100%).

3.3. HPLC method

A Merck LiChrospher 60RP select B (5 μm, 125 × 4 mm) column was used either on a Varian

5000 liquid chromatograph with a Waters 990 photodiode array detector, or alternatively on a Varian liquid chromatograph with a UV detector from Spectra Physics (Integrater SP 4270). The diisocyanate was weighed into a volumetric flask and made up to 100 ml with solvent. Aliquots of this solution were removed at set intervals and added to a solution of dibutylamine in acetonitrile. The NCO groups still present at the time in question react immediately with the dibutylamine to form the corresponding ureas, and are thus protected from further reaction with water.

3.4. Solvents for aromatic diisocyanates

Compounds tested for mutagenicity in the Salmonella/microsome test are generally dissolved in DMSO if they are insoluble in water. It was shown that less than 0.5 ml DMSO per plate did not interfere with mutagenesis, or with microsomal activity [14]. However, there have also been problems associated with the use of DMSO. For example, it readily picks up water, which can hydrolyse reactive compounds. Furthermore, DMSO is not chemically inert [29]. For these reasons, and also because some compounds are insoluble in DMSO, other organic solvents were screened for compatibility in the Salmonella/microsome test. Several solvents capable of dissolving a wide range of chemicals were found to be satisfactory under the specified conditions [16]. Some of the mentioned solvents (e.g., alcohols, amines, hydroxyethers) had to be ruled out because they can react with aromatic diisocyanates to form ureas or urethanes. Among the inert solvents mentioned—hydrocarbons and ethers—EGDE was chosen as an alternative solvent for the diisocyanates.

3.5. Strains

The Salmonella typhimurium LT2 mutants TA 1535, TA 100, TA 1537 and TA 98 were used. All strains were obtained directly from Bruce Ames and checked at regular intervals for their genotypic stability. Strains were stored DMSO protected as frozen permanents at about −80°C in 1 ml portions. They were thawed immediately prior to use. 0.2 ml was added per 10 ml nutrient broth, incubated and shaken overnight at 37°C and 90 rpm and used only on the same day.

3.6. Metabolic activation system

S9 fraction was obtained from Aroclor 1254 (500 mg/kg in corn oil, single intraperitoneal injection, 5 days prior to sacrifice) induced male Sprague–Dawley rats (200–300 g). After sacrifice livers were removed, washed (0.15 M KCl, 1 ml/g) and homogenised (0.15 M KCl, 3 ml/g) under sterile conditions at about 4°C. The 9000 g supernatant was stored at about −80°C. The required portions were slowly thawed, the S9 mix prepared and used only on the same day. The S9 mix was composed of 7 parts phosphate buffer [100 mM 162.6 mg $MgCl_2 \times H_2O$, 246 mg KCl, 179.1 mg glucose-6-phosphate (Na_2 salt), 315 mg NADP (Na_2 salt)] and 3 parts S9 fraction (30%) or 9 parts of phosphate buffer (100 mM) and 1 part S9 fraction (10%). The respective amount of S9 fraction used is indicated in Tables 5–7.

3.7. Salmonella / microsome test

The test, performed according to the plate incorporation method, followed the directions of Ames et al. [14] and Maron and Ames [15]. For the mutant count, at least three plates were used for each strain and dose. An equal number of plates containing the solvent minus the test substance comprised the negative control. Each positive control also involved at least three plates per strain. In experiments without S9 mix, buffer was used as replacement. The amount of solvent was 0.1 ml/plate.

The toxicity of the substance was assessed as a gross appraisal of background growth on mutant plates and/or as a marked and dose-dependent reduction in the mutant count.

The count was made after the plates had been incubated for 48 h at 37°C. If no immediate count was possible, plates were temporarily stored in a refrigerator. All results were confirmed in at least one independent experiment. A reproducible and dose-related increase in mutant counts of at least one strain is considered to be a positive result. For TA 1535, TA 100 and TA 98, this increase should be about twice that of negative controls, whereas for TA 1537 at least a threefold increase is required. How-

ever, these criteria may be overruled by good scientific judgement.

3.8. Fate of diisocyanates in Salmonella / microsome test environments

The procedure of the Salmonella/microsome test can be subdivided into different sections in which chemical reactions between the diisocyanate and water and possibly the functional groups of the biological test ingredients could take place. Two of these segments were simulated in the laboratory and checked for their content of aromatic diisocyanates and for possible reaction products.

In the first part, the test compound was dissolved in DMSO and in EGDE. The stability of these homogeneous solutions was monitored over a period of 4 h (ambient temperature/daylight). This duplicated a situation that may occur prior to the microsome test.

In a second series, the predissolved compound was mixed with an excess of water (37°C/daylight). The composition of the heterogeneous mixture was examined five times during the first minute of the test. These frequent analyses provided information as to what happened to the diisocyanate when it was mixed in a test tube with the aqueous test ingredients just before it was poured onto an agar plate. All this takes place within 60 s.

Twenty six milliliters of water, to which 1.0 ml of the dissolved compound was added, simulated the volume ratio of the test ingredients—2.0 ml of molten top agar, 0.1 ml of nutrient broth culture of the bacterial tester strain and 0.5 ml of S9 mix (or buffer)—which are all basically aqueous solutions. The use of water, instead of the complete set of ingredients, did not exactly duplicate, but was close to the conditions of the mutagenicity assays. The 10-fold approach simplified the evaluation of the chemical analyses to a great extent.

To prove the validity of the 'water model', two of the diisocyanates—2,6-TDI and 4,4′-MDI—were comparatively exposed to the real biological test environment. 0.1 ml of the (EGDE) dissolved compound was mixed with 2.6 ml of the test ingredients (2.0 ml agar, 0.1 ml nutrient broth, 0.5 ml S9 mix containing 10% S9 fraction) at 37°C/daylight. The composition of the reaction mixtures was again analysed five times within the first minute of the test.

4. Results and discussion

4.1. Stability of solutions of diisocyanates prior to Salmonella / microsome tests

The N=C=O content of 50–500 mg TDI, dissolved in 100 ml relatively 'dry' DMSO (0.02–0.03% water), dropped to 60% or less within the first 15 min of the test. Theoretically, the hydrolysis of 174 mg (1.0 mM) of TDI could consume 18 mg (1.0 mM) of water to form highly reactive intermediates, the aminoisocyanatotoluenes (TDAIs), and carbon dioxide. A homogeneous solution containing 0.02% (1.11 mM) water, as was the case for the 500 mg (2.86 mM) 2,4-TDI sample, would therefore convert about 40% of the TDI into reactive intermediates on a purely stoichiometric basis. These aminoisocyanates would then be available for further reactions with remaining 2,4-TDI or with themselves to produce a number of monomeric, oligomeric and polymeric ureas, which may be terminated by N=C=O and/or NH_2 groups. At this point, it is important to recall that a residual N=C=O content by no means indicates the presence of unmodified 2,4-TDI. The IR spectrum provides only insufficient information on the location of the N=C=O groups. The observed decline of isocyanate absorptions is, however, proof of the fact that chemical reactions have occurred.

In an additional experiment, 500 mg (2.86 mM) 2,4-TDI was dissolved in DMSO with an increased water content of 0.1% (5.56 mM), a level perfectly conceivable in practice. This amount of water led to an accelerated reduction of the N=C=O absorptions, so that after 15 min, only 43%, and after 4 h, 14% of the isocyanate groups could be detected.

The reaction patterns of MDI in 'dry' DMSO were comparable with those of TDI. These data have already been published [19].

Compared with the findings in DMSO, homogeneous solutions of aromatic diisocyanates in EGDE can be considered relatively stable. Even after 4 h, more than 95% of the N=C=O groups of 2,4-TDI still existed. Increasing the water content to an unrealistic 0.39% did not influence the stability of these solutions tremendously, although enough water was

available to convert all the N=C=O groups into amines and/or polymeric ureas. Solutions of MDI in EGDE behaved similarly to those of TDI.

As the IR method does not provide reliable information on the type and the number of reaction products formed, the stability of solutions of 2,4-TDI and 4,4′-MDI was additionally monitored by HPLC with ultraviolet (UV) detection. The advantage of this method is that the unreacted diisocyanates, as well as their possible reaction product can be quantified if suitable reference compounds are available.

501 mg (2.88 mM) of 2,4-TDI were dissolved in 100 ml DMSO that contained 0.04% (2.22 mM) of water. Fifteen minutes after the preparation of the solution, only 7% of the original 2,4-TDI were left over, and after 30 min, no more 2,4-TDI could be found at all. After 15 min, significant amounts of TDI-U, traces of 2,4-TDA, but no TDA-U were detected. The 2,4-TDA content was 0.8% after 30 min and rose to a constant level of 1.2% as the test proceeded. Absorptions of TDA-U first appeared after 30 min and increased continuously. Signals for the TDI-U passed through a maximum after 45 to 60 min and disappeared again within the 4 h of the test.

The reaction path of 4,4′-MDI in moist DMSO was very similar to that of 2,4-TDI. Only 1% of the unmodified 4,4′-MDI was left over after 30 min. 4,4′-MDA, with a final concentration of 3%, could be detected in solution almost immediately. Several multiple peaks appeared and created complex chromatograms resembling those of 2,4-TDI. Although no suitable reference compounds (MDI-ureas) were available, it seems reasonable to assume that MDI and TDI form comparable degradation products in moist DMSO.

The mode of degradation of 2,4-TDI and 4,4′-MDI, dissolved in EGDE, was different to that in DMSO, the main difference being that neither 2,4-TDA nor 4,4′-MDA were found. Furthermore, solutions with a nearly 3-fold surplus of water were comparably stable. After 1 h, between 98 and 99% of the original diisocyanates were still available and after 4 h, more than 85% could be found. In a supplementary experiment, the influence of increased amounts of water on the stability of solutions of 4,4′-MDI in EGDE was monitored. In a nearly equimolar solution (4.03 mM 4,4′-MDI: 3.89 mM water), more than 99% of the diisocyanate was still present after a period of 4 h. Raising the water content to 26.11 mM, which brought the diisocyanate:water ratio to approx. 1:7, left more than 93% of the original diisocyanate after 1 h, and still 79% after 4 h.

The HPLC analyses of the dissolved aromatic diisocyanates indicate that their degradation is considerably accelerated if DMSO is the solvent and may be completed before the Salmonella/microsome test has even begun. Among the various reaction products, traces of aromatic diamines can be detected. Taking this into account, we stopped the use of DMSO as solvent in bacterial tests. EGDE seems to be more suitable as it does not influence the stability of dissolved diisocyanates tremendously. TDI and MDI endure the dissolving and should therefore still be available for the tests if the solutions are used up within a few hours. No aromatic diamines can be discovered in this period of time.

It is suggested that the findings of previous workers on the mutagenicity of TDI and MDI are due to the use of moist DMSO and the results described are those expected for solutions that have generated traces of TDA or MDA [3,6,8]. The authors [3] have ascribed the mutagenic effects of TDI and MDI to the amine analogues (TDA and MDA) formed through the hydrolysis of the isocyanates. They were, however, not aware of the fact that the hydrolysis in DMSO (which leads to the complete disappearance of these diisocyanates within minutes) took place prior to the start of the Salmonella/microsome test. The solutions that were finally used in these tests contained mainly ureas and polyureas, besides traces of TDA or MDA, but practically no TDI or MDI. The authors [3] have also tested two prepolymerized types of TDI (Desmodur® L and Desmodur® E 1361), and found no mutagenic effects in the Salmonella/microsome tests. These results do not contradict the previous findings. As the two prepolymers used were virtually free of monomeric TDI (monomer content $< 0.5\%$), no monomeric TDA could be generated when they were dissolved in moist DMSO.

4.2. Fate of aromatic diisocyanates in simulated Salmonella / microsome tests environments

The sequential nature of the hydrolysis of aromatic diisocyanates has been described in Section 2. In consequence of this, the technique used for the

introduction and dispersal of TDI (or MDI) in aqueous media can significantly affect the chemical composition of the reaction products. Therefore, experimental studies of heterogeneous reactions of predissolved diisocyanates in a surplus of water were designed to enable a better prediction of their behaviour in toxicological tests. In the absence of biological macromolecules, competing reactions—especially those with NH_2 groups on proteins—do not occur.

The chemical changes that took place when TDI solutions in EGDE were added to an excess of water are summarised in Table 1. After 1 min, equivalent to the period within which the predissolved compounds should be mixed with water (or with the real test ingredients) and poured onto agar plates, the TDI contents of the 500 μg/test tube samples dropped to 45–35% of their initial concentrations. The strongest reductions, to as low as 57%, took place during the first 15 s. At the same time, signals indicating the presence of TDA, TDAI and TDA-U began to appear. The overall reaction led to a complex mixture containing TDI, TDAI, TDA, TDI-U, TDAI-U, TDA-U and others. Their composition varied with time. The maximum TDA concentrations observed were 3.9% after 15 s and 10.7% after 60 s. Thus, not the intended 500 μg TDI, but mixtures containing as little as 175 μg TDI, contaminated with up to 55 μg TDA and 270 μg of other products will be poured onto the plates. It may also be assumed that more, if not all, of the remaining TDI will react with water during the 48 h of incubation at 37°C. This part of the test, which leads to a further dilution of the reaction mixture, was not modelled in our experiments. However, in order to get a rough indication on the progress of the ongoing chemical reactions, the compositions of the mixtures were checked again after 1–2 days. No more TDI was then found in any of the samples. The TDA contents, however, remained more or less unchanged between the first minute and the 48th hour of the test.

The TDI contents of the 5000 μg/test tube samples dropped to 93–90% during the first minute (Table 1). At the same time, the various ureas as well as 0.65–0.98% TDA were formed. Therefore, the heterogeneous mixtures incubated did not consist of 5000 μg TDI/plate, but were blends of 4500 μg or more TDI, up to 49 μg TDA and further unquantified products. It is interesting to note that although the initial TDI concentrations in the two test series differed by one order of magnitude (500 vs. 5000 μg/plate), similar amounts of TDA were formed during these few seconds of mixing.

The behaviour of MDI was very different to that of TDI (Table 2). At both concentrations, more than 90% of the MDI was still available after 1 min. During this time, no MDA was detected in any of the 4,4′-MDI, MDI monomer mix or p-MDI runs. However, in one of the 2,4′-MDI isomer concentrations (500 mg/100 ml EGDE), 0.2 and 0.3% of 2,4′-MDA were found after 45 and 60 s, respectively. This indicates that the 500 μg 2,4′-MDI/test tube samples shaken with water for 45 or 60 s led to the deposition of traces of 2,4′-MDA on the plates. In all other MDI series, the incubation begins in the absence of MDA.

4.3. Fate of 2,6-TDI and 4,4′-MDI in real Salmonella / microsome test environments

Comparative experiments on the fate of 2,6-TDI and 4,4′-MDI in simulated and real Salmonella/microsome test environments were carried out. Again, five samples of each were taken within the first minute of the test and analysed for the residual amount of diisocyanates and for the yield of corresponding reaction products. The results are summarised in Tables 3 and 4.

When a sample of 500 mg 2,6-TDI/100 ml EGDE solution was mixed with the test ingredients or with water (0.1:2.6 ml), up to 6.6% of the TDI was converted into 2,6-TDA within 45 s (Table 3). With respect to the amount of diamine produced, no difference was seen between the distilled water and the aqueous system of the Salmonella/microsome test. Concerning the decline of diisocyanate content, however, the transfer to a different environment became apparent. In water, the 2,6-TDI concentration dropped to around 60%, which is basically in agreement with the results of the 10-fold approaches shown in Table 1 for the different types of TDI. When the ingredients of the Salmonella/microsome test replaced water, more than 90% of the initial 2,6-TDI disappeared within 15–45 s. In this case, not 500 μg, but less than 50 μg 2,6-TDI, enriched with around 25 μg

Table 1
Stability TDI (in EGDE) during the first minute of a simulated mutagenicity test[a]; HPLC determination of residual TDI and its reaction products

Diisocyanate	2,4-TDI				2,6-TDI				TDI 80			
TDI in 100 ml EGDE	5000 mg		500 mg		5000 mg		500 mg		500 mg			
Dose/plate	5000 μg		500 μg		5000 μg		500 μg		500 μg			
Analysed products[b]	2,4-TDI [%]	2,4-TDA [%]	2,4-TDI [%]	2,4-TDA [%]	2,6-TDI [%]	2,6-TDA [%]	2,6-TDI [%]	2,6-TDA [%]	2,4-TDI [%]	2,6-TDI [%]	2,4-TDA [%]	2,6-TDA [%]
Start	100	nd	100	nd	100	nd	100	nd	100	100	nd	nd
After 15 s	95	0.35	57	3.9	91	0.27	67	3.5	61	68	3.1	0.7
After 30 s	83	0.65	48	5.7	92	0.56	50	6.6	52	61	4.4	1.0
After 45 s	93	0.60	41	6.6	92	0.81	37	9.5	36	47	5.7	1.6
After 60 s	93	0.65	35	7.4	90	0.98	40	10.7	35	45	6.0	1.9

nd: Not detectable, detection limit: 0.1%, e.g., 0.5 μg for the 500 mg/100 ml concentration.
na: Not available.
[a]Simulating the mixing of dissolved TDI with the test ingredients (1 ml:26 ml mix with water).
[b]Ureas and (insoluble) polyureas were not quantified.

Table 2
Stability of MDI (in EGDE) during the first minute of a simulated mutagenicity test[a]; HPLC determination of residual MDI and its reaction products

Diisocyanate	4,4′-MDI				MDI monomer mix[b]							
MDI in 100 ml EGDE	5000 mg		500 mg		5000 mg				500 mg			
Dose/plate	5000 μg		500 μg		5000 μg				500 μg			
Analysed products[c]	4,4′-MDI [%]	4,4′-MDA [%]	4,4′-MDI [%]	4,4′-MDA [%]	4,4′-MDI [%]	2,4′-MDI [%]	4,4′-MDA [%]	2,4′-MDA [%]	4,4′-MDI [%]	2,4′-MDI [%]	4,4′-MDA [%]	2,4′-MDA [%]
Start	100	nd	100	nd	100	100	nd	nd	100	100	nd	nd
After 15 s	97	nd	95	nd	97	97	nd	nd	96	96	nd	nd
After 30 s	96	nd	95	nd	98	98	nd	nd	95	96	nd	nd
After 45 s	98	nd	94	nd	99	99	nd	nd	95	96	nd	nd
After 60 s	95	nd	91	nd	97	98	nd	nd	93	94	nd	nd

Diisocyanate	2,4′-MDI				MDI polymer			
MDI in 100 ml EGDE	5000 mg		500 mg		5000 mg		500 mg	
Dose/plate	5000 μg		500 μg		5000 μg		500 μg	
Analysed products[c]	2,4′-MDI [%]	2,4′-MDA [%]	2,4′-MDI [%]	2,4′-MDA [%]	4,4′-MDI [%]	4,4′-MDA [%]	4,4′-MDI [%]	4,4′-MDA [%]
Start	100	nd	100	nd	100	nd	100	nd
After 15 s	97	nd	97	nd	101	nd	101	nd
After 30 s	95	nd	97	nd	na	nd	104	nd
After 45 s	97	nd	95	0.2	99	nd	90	nd
After 60 s	97	nd	95	0.3	98	nd	95	nd

nd: Not detectable, detection limit: 0.1%, e. g. 0.5 μg for the 500 mg/100 ml concentration.
na: Not available.
[a] Simulating the mixing of dissolved MDI with the test ingredients (1 ml:26 ml mix with water).
[b] Mixture of isomers of monomeric MDI (4,4′-, 2,4′- and 2,2′-MDI).
[c] Ureas and (insoluble) polyureas were not quantified.

Table 3
Stability of 2,6-TDI during the first minute of the mutagenicity test[a]; HPLC determination of residual 2,6-TDI and its reaction products

2,6-TDI in 100 ml solvent	500 mg/EGDE		500 mg/EGDE		500 mg/DMSO	
Reaction medium	Dist. water		Test ingredients[b]		Test ingredients[b]	
2,6-TDI/plate	500 μg		500 μg		500 μg	
Analysed products[c]	2,6-TDI [%]	2,6-TDA [%]	2,6-TDI [%]	2,6-TDA [%]	2,6-TDI [%]	2,6-TDA [%]
Start	100	nd	99.5	0.5	12.3	9.1
After 5 s	77.8	1.6	23.1	1.6	2.3	6.4
After 15 s	70.0	3.4	8.4	4.7	3.0	8.4
After 30 s	60.7	5.3	5.6	5.8	2.6	9.1
After 45 s	61.9	6.6	8.1	5.6	2.5	8.3

nd: Not detectable; detection limit: 0.1%, e.g., 0.5 μg.
[a] Mixing 0.1 ml dissolved 2,6-TDI with 2.6 ml water or 2.6 ml test ingredients.
[b] 2.0 ml agar + 0.5 ml S9 mix + 0.1 ml nutrient broth.
[c] Ureas and (insoluble) polyureas were not quantified.

2,6-TDA and further unquantified products will be poured onto the plate.

Reactions of 4,4′-MDI and water or aqueous test ingredients were markedly slower than those of 2,6-TDI. No MDA was detected when the predissolved MDI was mixed with water (Table 4). Similarly, in the presence of the real test ingredients, no MDA was seen during the first 30 s of the test. However, after 45 s, 0,6% 4,4′-MDA appeared. At this time, less than 30% 4,4′-MDI remained intact, whereas in distilled water around 95% of the initial diisocyanate were still available for the bacterial test.

In summary, it has been shown that the ‘water model’ can be used for a general prediction of the chemical reactions that occur in the real test environment. In both cases, the water reaction of predissolved TDI was faster than that of predissolved MDI. Comparable amounts of aromatic amines were found for TDI and MDI in both test media. The accelerated disappearance of the TDI and the MDI in the presence of the complete set of test ingredients can be explained by the consumption of the aromatic diisocyanates through the rapid reactions with the biological macromolecules.

At the end of the analytical part of this study, the area concerning the stability of aromatic diisocyanates predissolved in DMSO was revisited to enable a more realistic comparison with EGDE. For this purpose, freshly prepared solutions of 2,6-TDI and 4,4′-MDI in DMSO were mixed with a complete

Table 4
Stability of 4,4′-MDI during the first minute of the mutagenicity test[a]; HPLC determination of residual 4,4′-MDI and its reaction products

4,4′-MDI in 100 ml solvent	500 mg/EGDE		500 mg/EGDE		500 mg/DMSO	
Reaction medium	Dist. water		Test ingredients[b]		Test ingredients[b]	
4,4′-MDI/plate	500 μg		500 μg		500 μg	
Analysed products[c]	4,4′-MDI [%]	4,4′-MDA [%]	4,4′-MDI [%]	4,4′-MDA [%]	4,4′-MDI [%]	4,4′-MDA [%]
Start	100	nd	100	nd	0.3	nd
After 5 s	99.6	nd	80.3	nd	0.2	2.8
After 15 s	99.1	nd	66.7	nd	0.7	2.8
After 30 s	94.9	nd	37.8	nd	0.3	2.7
After 45 s	95.5	nd	28.8	0.6	nd	2.1

nd: Not detectable; detection limit: 0.1%, e.g., 0.5 μg.
[a] Mixing 0.1 ml dissolved 4,4′-MDI with 2.6 ml water or 2.6 ml test ingredients.
[b] 2.0 ml agar + 0.5 ml S9 mix + 0.1 ml nutrient broth.
[c] Ureas and (insoluble) polyureas were not quantified.

set of the aqueous test ingredients. The chemical composition of the mixtures was monitored over a period of 45 s. As shown in Tables 3 and 4, the content of 2,6-TDI and 4,4′-MDI disappeared almost completely within the first minute of this test. Only around 3% of the 2,6-TDI and less than 0.7% of 4,4′-MDI were left over in solution. At the same time, up to 9.1% 2,6-TDA and 2,8% 4,4′-MDA appeared. These findings explain the positive Salmonella/microsome test results reported for TDI [3] and four types of MDI [19], all of which were predissolved in DMSO.

From the comparison of DMSO and EGDE as solvents for aromatic diisocyanates in bacterial tests, the conclusion can be drawn that the chosen solvent considerably influences the biochemical fate of reactive chemicals. Tables 3 and 4 illustrate convincingly that the chemical composition of the test compounds in DMSO is different to that in EGDE and this difference may be the reason for the contradicting results reported in the past.

4.4. Salmonella / microsome tests

The data for 4,4′-MDI, 2,4′-MDI, the mixture of monomeric MDI isomers and polymeric MDI have already been published [19]. In summary, all compounds revealed mutagenic effects with S9 mix when they were dissolved in DMSO, whereas no mutagenic effects were observed in EGDE with or without S9 mix.

Table 5
Results with 2,4-TDI (dissolved in EGDE) and metabolic activation

Strain S9, μg/plate	TA 1537		TA 98	
	10% S9	30% S9	10% S9	30% S9
0	19	12	54	39
50	18	16	64	52
100	23	16	104∗	76∗
200	44∗b	25	117∗	76∗
400	52∗b	22b	153∗b	87∗b
600	51∗b	45∗b	172∗b	93∗b
800	49∗bp	41∗b	125∗bp	128∗bp
1000	41∗bp	42∗bp	117∗bp	58∗bp
AA 3	233∗	80∗bp	1245∗	509∗

AA = 2-aminoanthracene, ∗ = mutagenic effect, b = background growth reduced, p = precipitation.

Table 6
Results with 2,6-TDI (dissolved in EGDE) and metabolic activation

Strain S9, μg/plate	TA 1537		TA 98	
	10% S9	30% S9	10% S9	30% S9
0	12	14	47	45
150	13	21	67	57
300	13	16	87∗	61
600	15	15p	130∗	73∗
1200	7p	10p	166∗p	97∗p
2400	7p	9p	128∗p	82∗p
4800	p	p	p	p
AA 3	360∗	66∗	1538∗	618∗

AA = 2-aminoanthracene, ∗ = mutagenic effect, b = background growth reduced, p = precipitation.

Published data on the mutagenicity of 2,4-TDI [8], 2,6-TDI [8] and TDI 80 [3,8] were based on the use of solutions in DMSO. In these experiments all three compounds showed uniformly negative results in the absence of S9 mix, while with S9 uniformly positive results were obtained for Salmonella typhimurium TA 100 and TA 98.

In the present investigations, 2,4-TDI, 2,6-TDI and TDI 80, all of which were dissolved in EGDE, showed a consistently negative response in the absence of S9 mix. Furthermore, no mutagenicity was observed in any of the TA 1535 strain experiments. With respect to these findings, the results of the TDIs were in complete agreement with those obtained for the MDIs. In contrast to the MDIs, however, clearly positive results were obtained in the other strains after metabolic activation of 2,4-TDI, 2,6-TDI and TDI 80. The results are summarised in Tables 5–7. Consistently positive results were ob-

Table 7
Results with TDI 80 (dissolved in EGDE) and 10% S9 mix

Strain, μg/plate	TA 100	TA 1537	TA 98
0	71	9	36
125	143	12	86∗
250	188∗	15	101∗
500	211∗p	21p	123∗p
1000	57p	28∗bp	88∗p
2000	21bp	2bp	23bp
AA 3	869∗	306∗	888∗

AA = 2-aminoanthracene, ∗ = mutagenic effect, b = background growth reduced, p = precipitation.

tained in strain TA 98 with all types of TDI tested. In TA 1537, clearly positive results were obtained for 2,4-TDI (Table 5) and weak effects were observed for TDI 80 whereas no effects were found for 2,6-TDI (Tables 6 and 7). Weak positive results were also obtained for TDI 80 with TA 100 (Table 7). S9 mixtures with varying amounts of the S9 fraction (10 and 30%) were used for 2,4- and 2,6-TDI. The results demonstrate (Tables 5 and 6) that effects are slightly reduced in the presence of the S9 mix containing 30% of the S9 fraction. This reduction of effects may be explained with a selective or at least preferred reaction of the test samples with the proteins of the S9 fraction. The 30% S9 mix contains three times as many proteins as the 10% S9 mix.

The positive test results described for TDI—and also the negative findings reported for MDI [19]—are in good agreement with the chemical analyses that monitored the fate of these reactive compounds through the different stages of the Salmonella/microsome test (Sections 4.2 and 4.3). As shown in Tables 1–4 the instability of solutions of TDI and MDI in the aqueous test environment leads almost immediately to the formation of a variety of reaction products. The composition of this product mix influences the outcome of the bacterial tests. The positive findings for three types of TDI can be explained with the appearance of TDA—a known mutagen—that is one of the reaction products generated when solutions of TDI in EGDE are added to the aqueous test system (Table 1). The negative results obtained for four types of MDI are due to the fact that no or no relevant amount of MDA is found in the test system.

5. Conclusions

Finally it can be concluded that predissolved aromatic diisocyanates react with the aqueous biological test media while the tests are going on. During the tests, TDI and MDI are converted into mixtures of chemical compounds similar in structure but different in composition. It is assumed that the Salmonella/microsome test results of TDI and MDI in reality do not reflect the properties of these products, but rather those of the compounds formed under the specific conditions of the test.

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