HALOGENATED SOLVENTS INDUSTRY ALLIANCE, INC.

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May 3, 2001

Dr. C. W. Jameson
National Toxicology Program
Report on Carcinogens, MD: EC-14
P. O. Box 12233
Research Triangle Park, NC 27709

Re: Comments on Proposal to List Trichloroethylene in the NTP Report on Carcinogens, Tenth Edition

Dear Dr. Jameson:

Please find enclosed the latest comments to be filed by HSIA in relation to the proposal to reclassify the carcinogenicity of trichloroethylene.

I also enclose the comments of Dr Bloemen that were supplied to NTP at the BSC meeting on December 13, 2000. I am sending these because Dr Bloemen's comments have not been made available, as yet, on the NTP web site and they do make points not recorded by any other commenter. The recent paper by Bloemen et al (2001) is also enclosed. This paper contains highly relevant information from a study involving human volunteers exposed to trichloroethylene under controlled conditions.

Yours sincerely,

Paul H. Dugard, PhD
Director of Scientific Programs

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Comment on Proposal to List Trichloroethylene in the NTP Report on Carcinogens, Tenth Edition

Dear Dr. Jameson:

The Halogenated Solvents Industry Alliance, Inc. (HSIA) represents the producers and users of chlorinated solvents, including trichloroethylene. Trichloroethylene was first listed as a chemical "reasonably anticipated to be a human carcinogen" in NTP's Ninth Report on Carcinogens, and this solvent has now been nominated for an upgraded listing to "known to be a human carcinogen" in the Tenth Report. HSIA considers that the scientific evidence does not justify the proposed upgrading, and requests that NTP withdraw the proposal to change the classification for trichloroethylene in the Tenth Report. It should be noted that this was the position (no change in classification) reached by the Board of Scientific Counselors Report on Carcinogens Subcommittee (BSC) on a vote of 9 to 1 at a meeting on December 13, 2000.

Since the nomination to upgrade the classification of trichloroethylene was first published (65 Fed. Reg. 17889; April 5, 2000) extensive public comments have been filed covering many aspects of the proposal. The document that follows will not duplicate the details provided in those earlier comments, but will make reference to them in providing an overview of the scientific issues. In addition to scientific issues, there are several procedural and general concerns noted by HSIA and these will be summarized.

Comments on Scientific Issues

1. Epidemiology

Epidemiological evidence is of primary importance in the carcinogenicity classification of an agent, and this is particularly true when the class under consideration is "known to be a human carcinogen".

1.1 Kidney Cancer

It is clear that the proposal to upgrade the classification of trichloroethylene is heavily dependent upon two studies of renal cell carcinoma:

Henschler et al (1995) [1] reported the incidence of kidney cancer at a cardboard factory. The authors have acknowledged subsequently that this was a study of an already recognized cluster. Therefore, by the established rules of epidemiological practice, this study cannot be used as evidence of an association between trichloroethylene and renal cell cancer; its role is limited to "hypothesis setting". The use of this study for classification purposes was severely criticized in a number of previous comments (Dugard, June 5, 2000; Mandel et al, June 5, 2000; Dugard, December 1, 2000; Adami and Trichopoulos, December 1, 2000; Maull, December 1, 2000: Lash et al, December 1, 2000; Mandel, December 1, 2000; Bloemen, December 13, 2000).
The comments of Dugard (June 5, 2000) showed that the pattern of kidney cancer in Henschler et al (1995) was much less likely to be associated with exposure to trichloroethylene than the authors indicated. Dr. Sheila Zahm[2], a member of the BSC and one of the two appointed reviewers for trichloroethylene, also expressed different concerns regarding the use of the Henschler et al study as a basis for classification and concluded "So there's just enough issues about that I wouldn't want to hang my hat on it and neither did IARC and neither did NTP during the last review". Dr. Zahm is Deputy Director of the Division of Cancer Epidemiology and Genetics, National Cancer Institute.

Vamvakas et al (1998) is a relatively small case control study purporting to show an association between high exposures to trichloroethylene and the incidence of renal cell cancer. Significant methodological flaws in this study have been recognized and detailed in the scientific literature (Green and Lash, 1999) and in comments related to NTP's proposal to change the classification of trichloroethylene (Mandel et al, June 5, 2000; Dugard, June , 2000; Adami and Trichopoulos, December 1, 2000; Maull, December 1, 2000; Lash et al, December 1, 2000; Mandel, December 1, 2000; Dugard, December 1, 2000; Bloemen, December 13, 2000). The results of this study suggest a strong association between trichloroethylene and kidney cancer that is at odds with the findings in large, well-conducted cohort studies such as Blair et al (1998) and Boice et al (1999) and their predecessors. According to Vamvakas et al, the reason for the difference is the magnitude of exposures. However, as discussed by Bloemen (December 13, 2000), these differences do not exist in reality. Mandel (November 29, 2000) shows that the poor methodology employed by Vamvakas et al could result in false odds ratios as high as those reported. Dr Zahm in her report to the BSC acknowledged the methodological flaws of Vamvakas et al (1998) and accorded this study little weight in comparison with the large, well conducted cohort studies, especially that by Blair et al (1998).

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[1] References may be found in the Background Document for trichloroethylene or are listed at the end of this document. Public Comments filed previously are characterized by author(s) and date of receipt shown by NTP, or the approximate date of submission to NTP.

[2] BSC member quotes and opinions may be found in the transcript of the December 13 meeting available from NTP.

Perhaps the bottom line on these two studies is best captured by the eminent, internationally recognized academics, Professors Adami and Trichopoulos (December 1, 2000): "These studies are, however, so methodologically flawed that they do not even meet basic quality criteria for a modern epidemiologic investigation. Hence we suggest that they be disregarded in the current evaluation process – at least until the original data have been scrutinized in detail by external reviewers."

With the exception of the two studies discussed above, the pattern of kidney cancer incidence across a number of occupational cohort studies is typical of the situation where no causality exists (Adami and Trichopoulos, December 1, 2000; Mandel, December 1, 2000; Lash et al, December 1, 2000).

1.2 Other Cancer Endpoints

The Background Document reviews findings in discreet studies and reviews. The numerical information from one recent review in particular, that of Wartenberg et al (2000) has been presented in detail. This review has been criticized (Bloemen, December 13, 2000,) for including data from the Henschler et al (1995) study in aggregating kidney cancer information from "Tier I" cohort studies and also because the "heterogeneity" of the studies has not been taken into account. Wartenberg et al (2000) acknowledge the heterogeneity of the studies but use a mathematical procedure that assumes homogeneity. Dr. George Bonney, the second BSC reviewer for trichloroethylene, stated regarding the approach of Wartenberg et al (2000) that "It's the kind of average that gives statistics a bad name". None of the published reviews of trichloroethylene epidemiology (Weiss, 1996; IARC, 1995; McLaughlin and Blot, 1997; Wartenberg et al, 2000) concluded that there is evidence for a causal relationship between trichloroethylene exposure and any type of cancer. Furthermore, the reviews found no convincing evidence of an association between trichloroethylene and any type of cancer. None of the authors of individual epidemiology studies identifies any causal relationships. In her analysis for the BSC, Dr. Zahm concluded that the new epidemiological information emerging since the IARC review, or NTP's listing of trichloroethylene in the Ninth Report, does not provide sufficient evidence to change the NTP listing to that of "known human carcinogen".

1.3 Conclusion

Despite the large number of studies, no clear association between any cancer type and exposure to trichloroethylene has emerged, and certainly no causal relationship.
Dr Zahm's analysis and conclusions were supported by Dr Bonney and other members of the BSC. Clearly, by the vote of 9 to 1 in favor of Dr Zahm's conclusion, the BSC supported her bottom line: ".....I recommend that it remains, at this point, as reasonably anticipated to be a human carcinogen".

2. Animal Data and Mechanisms of Action

2.1 Results of Carcinogenicity Assays

Although the Background Document identifies a variety of tumors whose incidences appeared to increase as a result of trichloroethylene administration, the only tumor types that should receive consideration are mouse liver and lung tumors and rat kidney tumors (Dugard, December 1, 2000).

2.2 Mouse Lung Tumors

In inhalation studies, the increase in incidence of lung adenomas/adenocarcinomas in certain strains of mice was not seen in any of several comparable studies in rats. The evidence is strong that this is a "mouse only response" (Green et al, 1997a; Green, 2000) and this is discussed in the Background Document.

2.3 Mouse Liver Tumors

Administration of trichloroethylene via oral or inhalation routes has been shown to increase the incidence of hepatocellular adenomas and/or carcinomas in certain mouse strains. No increase in liver tumors has been observed in any rat study. The Background Document places too great an emphasis on the possible role that dichloroacetic acid (DCA) may play in the development of these tumors because this material is a very minor metabolite of trichloroethylene (Dugard, December 1, 2000; Barton et al, 1999; Merdink et al, 1998). The role of peroxisome proliferation in the induction of mouse liver tumors is addressed in the Background Document. It is likely that trichloroacetic acid (TCA) interacts with the receptor, PPARα, to induce the peroxisome proliferation response. It is considered that events associated with the activation of PPARα, such as cell proliferation and reduced apoptosis, underlie the induction of rodent liver tumors by peroxisome proliferators (Chevalier and Roberts, 1998). Even potent peroxisome proliferators in rodents have not been found to induce, in human liver cells, the responses associated with tumor induction. Long-term use of hypolipodemic drugs that are potent peroxisome proliferators and liver carcinogens in rodents has not been associated with any increase in cancer in human patients. Hence, US FDA does not regard rodent liver tumors in association with peroxisome proliferation to be a concern for carcinogenicity in humans. Dugard (December 1, 2000) offered an appropriate conclusion to the section on liver tumors in the Background Document:

"The evidence is accumulating to support the view that TCA induces mouse liver tumors via a non-genotoxic mechanism involving PPARα and gene expression leading to specific responses such as cell proliferation. The role of DCA is now considered to be minor to negligible in the induction of mouse liver tumors by trichloroethylene. There is growing evidence that man does not respond to peroxisome proliferators in a manner that might lead to liver tumors. Therefore, the probability is high that the liver tumors

induced in mice by trichloroethylene are not indicative of potential human carcinogenicity. (This is the view held by European and Canadian regulatory authorities)."

2.4 Rat Kidney Tumors

The increases in kidney tubular adenoma and/or adenocarcinoma, alleged to have been induced by trichloroethylene, are very small in magnitude, and confined to the males of certain rat strains only. The most recent evidence relating to the potential origins of these tumors was discussed in Dugard (December 1, 2000) and Green (November 30, 2000): Rats treated with trichloroethylene in long term studies experience prolonged kidney damage. Until recently, a plausible hypothesis was that a minor metabolite of trichloroethylene, S-(1,2-dichlorovinyl)-L-cysteine (DCVC) is activated by a renal enzyme, β-lyase, and that the reactive products generated cause tubular damage. It has been presumed that the prolonged tubular cell damage was essential for tumor formation. DCVC has not been found to be genotoxic in the kidney, and, in a limited study, was not a potent rat kidney carcinogen (Terracini & Parker, 1965). The role of DCVC now has to be re-evaluated. Although DCVC is known to damage the rat kidney, the rat simply does not generate sufficient DCVC to explain the kidney damage observed in trichloroethylene experiments – the level generated is three orders of magnitude below the no effect level established for DCVC itself (Green, 1997b). Green et al (1998) have established that the source of kidney damage is formic acid that is excreted in high levels by rats exposed to trichloroethylene. There is growing evidence that the formic acid mechanism is not relevant to humans (Green et al, in preparation) and the levels of DCVC generated by man from trichloroethylene are even further below levels at which kidney damage could be expected (Bernauer et al, 1998; Bloemen et al, 2001).

It was concluded in Dugard (December 1, 2000): "In sum, the once plausible hypothesis that kidney toxicity and tumors in rats exposed to trichloroethylene arose through renal activation of DCVC is no longer tenable now that more is known of the biological effects and potency of DCVC, and the levels produced from trichloroethylene."

2.5 Conclusions

The current understanding of the mechanisms responsible for tumors induced by trichloroethylene in long-term experiments in rodents shows that it is unlikely that these tumors are relevant to humans. It is clear these effects in animals cannot be used to force an interpretation from uncertain epidemiological evidence.

3. <u>The Role of the von Hippel-Lindau (VHL) Gene</u>

Comments filed by Gnarra (December 1, 2000) summarize what is known about the relationship between the VHL tumor suppressor gene and renal cell cancer. The majority (75 – 80%) of human renal cell cancer is of the clear cell type and, in 70 – 80% of these cancers, the VHL gene is somatically inactivated. Brauch et al (1999) claim to have found a greater number of multiple mutations of the VHL gene in renal cell cancers from

patients having been exposed to trichloroethylene at high levels, and a "hot spot" mutation present only in exposed patients. Gnarra (December 1, 2000) identifies several concerns regarding this study and its interpretation in the Background Document. One concern is that multiple mutations in a single gene would not be expected (a very large number of mutations throughout the genome would have to occur, for example) and multiple mutations would not lead to the clonal advantage necessary for a cell to develop into cancer. Brauch, herself, has published a paper (Brauch et al, 2000) that shows that later stage renal cell carcinomas have multiple mutations of the VHL gene. The stage of the tumors in trichloroethylene exposed patients was not displayed in Brauch's earlier paper.

The "hot spot" mutation, if specific for trichloroethylene, is potentially very significant. However, Gnarra (December 1, 2000) identifies significant concerns regarding the conduct of the Brauch et al (1999) study, and confirmation of the "hot spot" finding is essential. The first such test (Schraml et al, 2000) failed to confirm Brauch's findings.

In reviewing the evidence regarding the VHL gene and trichloroethylene, the BSC arrived at similar conclusions to those of Gnarra (December 1, 2000). Drs Zahm and Phillips referred to previous specific "hot spot" findings that have not been reproducible. The need for confirmation of the "hot spot" finding appeared to be supported by a majority of the BSC.

4. General Conclusions

Perhaps the summary of his views by Dr Hilary Carpenter captured the feelings of many of the BSC. He came to the meeting "on the fence with trichloroethylene" but "...wanted to be swayed toward public health." However, "I think that the evidence at this point that's been presented by the groups this morning and discussions indicate that we really don't have enough information to consider it as a known carcinogen." Dr Zahm's motion that the listing of trichloroethylene remain as reasonably anticipated to be a human carcinogen was carried by a BSC vote of 9 to1.

General Issues

NTP Provided Inadequate Notice and Information to Allow Informed Public Comment

HSIA's comments of June 5, 2000 (Dugard, June 5, 2000), not repeated here, discuss the ways in which the process failed to provide sufficient basis for public comment. In addition, as indicated in HSIA's subsequent comments (Dugard, December 1, 2000), the Background Document has serious shortcomings as a scientific document that should have presented comprehensive, up-to-date, accurate information reported without bias. In the event, the Background Document fell far short of meeting any of these fundamental criteria.

Because the Background Document was only made available for public comment in mid-October 2000, public comments sought in the Federal Register notice of April 5, 2000 could not be based on any scientific case – the reasons for nominations not being displayed. Moreover, the October release of this document allowed only a very short period of time for public comments to be filed and, even worse, meant that the Board of Scientific Counselors RoC Subcommittee had an impossibly short period (less than two weeks) in which to consider comments by the public on nominations to be discussed at their meeting.

The Background Document was written by a contractor, and it is possible that a draft was available early in 2000 to the NIEHS staff who comprised the RG 1 committee. With this treatment of the science coloring judgment, it is, perhaps, not surprising that RG 1 voted unanimously to support their own nomination for upgrading the classification of trichloroethylene. It is disappointing that the well-qualified scientists of NIEHS failed to recognize the many shortcomings of the Background Document and request substantial revisions.

Respectfully submitted on behalf of the Halogenated Solvents Industry Alliance, Inc. by

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Director of Scientific Programs

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Main points of concern on the National Toxicology Program Draft Background Document for Trichloroethylene.

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The criteria for listing Agents, Substances or Mixtures in the Report on Carcinogens stipulate that consideration should be given to all relevant information. The background document completely and systematically ignores the descriptions of historical worker exposure, given extensively for instance in the IARC Monograph No. 63 or the publication by Stewart et al. (1991). In doing so, the document does not meet the criteria for an NTP Background Document. Scientific judgment based on this document can easily be flawed, as is demonstrated below.

The plausibility of the risk of 10 for renal cell cancer seen in the studies by Henschler et al (1995) and Vamvakas et al (1998) depends on the argument of exceptional exposure levels experienced by the study populations. There is ample information the literature which makes clear that use of trichloroethylene described in these studies was consistent with contemporary procedures in general. Hundreds of thousands of workers have been using trichloroethylene this way for many years (IARC 1995). A risk of this size cannot be missed and certainly would have shown up, if not in registry based studies. In reality, no increased risk for renal cell cancer has been seen after eighty years of intensive use of trichloroethylene.

The background document extensively quotes the meta-analysis publication by Wartenburg et al. (2000). This quotation however is biased by only mentioning the results, and ignoring the careful comments from the authors. The comments and suggestions show clearly that the results of the analysis were not definitive, even in the authors' eyes.

The approach used to aggregate the results of the different epidemiological studies used by Wartenburg et al. (2000) is inappropriate for this situation. Many of the required considerations for meta-analysis that were defined in an earlier paper (Blair et al 1995) that includes Wartenberg as a co-author, are ignored here. Wartenberg et al. do not address heterogeneity or differences in exposure levels between studies, but only weigh studies according to precision, which is related to cohort size. Cohort size is only one of the determinants of study relevance. Wartenburg's analysis ignores the negative results revealed by the internal analyses in these studies: risk related to duration of exposure or dose. These omissions make the meta-analysis incorrect and misleading.

The large cohort studies compare disease or mortality patterns in the work force with patterns seen for the general population at country or at best, at state level. The small differences seen in these comparisons are much more credibly explained by bias and confounding, because of comparing unmatched groups, than by exposure to trichloroethylene. The lack of consistent patterns when looking for association with duration of exposure or level of exposure, makes this point very clear.

Completeness of information.

The Criteria for listing Agents, Substances or Mixtures in the Report on Carcinogens by the U.S. Department of Health and Human Services national Toxicology Program, as given in the Draft Background Document for Trichloroethylene, clearly state:

"Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information."

The Background Document explains the use of trichloethylene in seven lines (2.1), indicates an annual production capacity in the US of 160,000 tons in a certain year (2.2) and gives a table which indicates that 400,000 workers were potentially exposed frm 1980 to 1983, 90,000 in the electric and electronic equipment industry and 30,000 while manufacturing fabricated products. This information underlines the point that trichloroethylene is a very important solvent and has been used extensively.

In contrast, no description of use of trichlorothylene and no measurements of workers' exposure are given. Not that this information does not exist. The IARC Monograph on trichlorothylene mentions the results of occupational air and biomonitoring results in reported in a large number of papers.

The systematic ignoring of information on exposure goes so far that the separate publication on the exposure situation in the most important cohort where highest exposures have occurred (Blair et al. 1998) has not even been mentioned and does not appear in the literature list (Stewart et al. 1991). This indicates a clear lack of understanding by the authors of the Background Document of the role of exposure in the interpretation of epidemiology studies.

Clear understanding of the historical use of a chemical and the exposures related to this allows the findings of the occupational studies to be put into context. The lack of this insight prevents readers of the Background Document from getting the proper

perspective and to arrive at sound scientific judgement on the carcinogenicity of trichloroethylene.

Historical use of trichloroethylene and levels of exposure

In a discussion on the health effects of exposure to trichloroethylene (TRI), it is important to consider historical use of TRI, and what exposure levels this resulted in.

The extensive information contained in Patty's Industrial Hygiene and Toxicology and the 1995 IARC Monograph 63 on Dry Cleaning, Some Chlorinated Solvents and Other Industrial Chemicals serve as the main source for the following description.

Commercial production of TRI started in 1920's in Germany and the US. It has been produced at a large scale in many industrialised countries. Manufacturing quantities range from 277 thousand tonnes in 1977 in the US and 210 thousand tonnes in Western Europe in 1980, to 60 thousand tonnes in Canada in 1976.

The major use of trichloroethylene is in metal degreasing. It is an excellent solvent for organic matter, it is non-flammable and relatively inexpensive (Grandjean 1955). Degreasing is important in all metalworking and maintenance operations to remove oils, greases, waxes, tars and moisture before final surface treatments such as galvanizing, electroplating, painting, anodizing and application of conversion coatings. Trichloroethylene is used in degreasing operations in industrial groups such as fabricated metal products, electric and electronic equipment and transport equipment.

Metal cleaning operations are of two types: cold cleaning and vapour cleaning. In cold cleaning, trichloroethylene is applied at room temperature; in vapour degreasing, the solvent vapours are condensed on the part to be cleaned. In cold cleaning, metal parts are either dipped in to the solvent solution or the solution is sprayed and wiped onto the object. The cold process is frequently used in maintenace operations and on small

parts. The manufacture of metal parts using a lathe, includes frequent dipping of the part into trichloroethylene to remove the cutting oil before measuring its dimensions. Trichloroethylene subsequently evaporates from the parts. Vapour degreasing requires a tank with heating coils on the bottom and a condensing zone near the top. The solvent is heated to boiling (87 ^{0}C), and the hot vapour fills the condensing zone near the top of the tank. Soiled objects are lowered into this zome, where the vapour condenses into a pure liquid solvent on the piece and dissolves and carries off dirt as it drains into the tank. The part dries immediately in the air. The tanks started to get equipped with cooling rings at the top reduce loss of the solvent and reduce exposure at the end of the 70's. Lids were also introduced to obtain closed machines, which are the rule nowadays. The effect of these measures can be seen on the annual use of the solvent in the US for metal cleaning: in 1971 trichloroethylene use for metal cleaning was 200,000 tonnes, this reduced to 84,000 tonnes in 1980.

Exposure levels up to the 80's had average levels of 60 ppm for eight hours and peaks often up to 400 ppm but occasionally up to 1000 ppm (IARC 1995). The TLV applied in the US in the 50's was 200 ppm, and in the UK it was 400 pm (Grandjean et al. 1955).

The smell of trichloroethylene has been described as not unpleasant, etherlike and deliberate inhalation of vapors by workers has been reported repeatedly. At room temperature, air saturation with trichloroethylene occurs at 70.000 ppm. Laboratory experiments have indicated that no effects are seen in man at exposures up to 100 ppm, marginal effects at 200 ppm and slight effects above 300-400 ppm. Eye and nasal irritation are the main adverse effects at or above these levels while CNS effects occur at levels of 1000 ppm or higher.

All this indicates that historical use of trichloroethylene has resulted in considerable levels of exposure for the workers to trichloroethylene. The inclusion of 106 cases of trichloroethylene poisoning in the Finnish cohort of 2198 workers studied by Tola et al (1980) and later by Antilla et al.(1995), and 32 cases of poisoning by trichloroethylene reported during the study period by Malek et al. (1979) underlines

this point. Fatalities from industrial exposure to trichloroethylene might be expected to have occurred in this time period and, indeed, a number have been reported (for overviews see Von Oettingen 1955, NIOSH 1973).

This is the background against which the cohort studies must be seen. In In the studies by Axelson (1995) and Antilla (1995), the cohorts were defined based on lists of participants in biomonitoring in during respectively 1955-1975 and 1965 –1982. This ensures inclusion of only people with the potential exposure to trichloroethylene. The investigators then used the results of the biomonitoring program to estimate exposure for the working period of the members of the cohort and conclude exposure levels have been rather low, less than 20 ppm for most of the Axelson cohort. This clearly is incorrect, only a few samples have been collected for each cohort member, no sampling procedure is given while the working period before the start of the study is ignored. In particular against the background of the way trichloroethylene was used, it is more resonable to expect that exposure of the two cohorts was much higher than assumed by the investigators. The inclusion of 106 cases of poisoning by trichloroethylene in the Antilla paper supports this notion.

Morgan et al. (1998) rate exposures low medium and high where work on degreasing machines was classified as high with exposures stated to be above 50 ppm. Boice at el. (1999) only describe use of trichloroethylene for vapour degreasing. For the initial study of the cohort reported on by Blair (Spirtas et al. 1991), extensive historical exposure assessment was performed which resulted in a separate publication (Stewart et al.1991). In the report, regular cleaning jobs were reported with exposure levels up to 600 ppm, in accordance with what is described above on usage of trichloroethylene.

The only other studies on trichloroethylene which give detailed information on exposure are the studies by Henschler et al (1995) and Vamvakas et al (199 . After identifying a cluster of kidney cancer cases in a cardboard factory descriptions of working practices 20 years ago involving bi-weekly cleaning of machinery using trichloroethylene must have impressed the investigators. They failed to note that cardboard factories are notoriously unpleasant to work in because of the formation of

hydrogen sulphide along with other organic sulphur compounds, all of which have an unpleasant, strong smell. The most common of these are methylmercaptan, ethylmercaptan, dimethylsulphide and hydrogen sulphide (IARC 1981). These circumstances are a better explanation for the compliants of the workers. The use of trichloroethylene for the bi-weekly cleaning job, done according to practices normal for that time period, will undoubtedly have contributed to the feelings of distress of the workers involved, but there is no reason to expect the working situation in the cardboard factory to be very different from that in other industries in that period. This is confirmed by historical reassessment of exposures in the publications on cohorts exposued to trichloroethylene by Cherrie et al (accepted for publication, J Clin Oncol and Cancer Research).

Vamvakas et al. (1998) expand the assumption of extremely and uncharacteristically high exposure levels from the cardboard factory to the area of the source population for their study, the area around a country hospital in North Rhine Westphalia, without any justification. They explain that exposures took place predominantly in small premises decades ago. There will undoubtedly have been high exposures amongst the study subjects but there are no reasons to expect the exposure situation to be very different from that of other users.

The argument that very high exposures explain the very aberrant findings in these two studies is not based on any data. Problems with study design and reporting made Adami and Trichopoulos (submitted to NTP) decide that these studies should not considered for regulatory evaluations.

Meta-analysis

Meta-analysis, other than for clinical trials, still is a subject of often heated discusison amongst epidemiologists. Calculating overall risk estimates based on combined raw data, pooling of data, or averaging risk estimates for individual studies is not contentious for experimental clinical trials because of good documentation, very similar study design and dosing strategies for these studies while minumal risk exists for bias and confounding because of the randomized study design.

No concensus exists on methods to obtain a numerical expression of risk seen in observational studies. Many epidemiologists consider that attempting to obtain a numerical results often requires multiple assumptions and therefore the outcome is misleading. The meta-analysis by Wartenburg et al. (2000) has generated an overall risk estimate with confidence intervals for several end-points, based on 7 cohort studies. These studies report on 20,000 workers exposed to TRI since the 50's. Others have reviewed these studies and concluded they concur with a null effect (Mandel et al. submitted for this meeting), while others have made very clear that the studies by Henschler et al. (1995) ad Vamvakas et al. (1999) are of such a low standard that they should not be considered for risk evaluation (Adami & Trichopoulos submitted for this meeting).

The discrepancy between the overall findings by Mandel et al and Wartenburg et al. (2000) can easily be explained by the inapprapriate method used by Wartenburg et al. Wartenburg is co-author of a recent paper on meta-analysis in environmental epidemiology (Blair et al. 1995) where a group of experienced epidemiologists give advice on how to conduct these studies. Wartenburg et al. mention some of the considerations from this paper, but in practice many important considerations are completeley ignored.

The main concern when merging study results is that of adding apples and oranges, adding studies that are different. Blair et al. state: "consideration of heterogeneity is

central to the decision of whether summary statistics should be produced in a meta-analysis and if so, how they should be produced (e.g. by stratifying the studies by the source of heterogeneity and conducting separate meta-analyses on the different subgroups)".

The paper by Wartenburg et al. shows no attempt to analyze heterogeneity, so heavily emphasized in his own earlier paper. To calculate the summary statistic, they use the inverse-study-variance method which is based on the the assumption of homogeneity.

Differences in exposure levels experienced by the different cohorts, would be a very good reason not to add studies. Indications for important differences are that Ritz et al. where trichloroethylene is used as a solvent, report only low to moderate exposures while the paper, focussed on exposure assessment of the aircraft maintenance cohort suggests jobs with regular exposure levels up to 600 ppm.

By ignoring these differences, Wartenburg et al. develop an incorrect and misleading summary statistic.

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Study on the cytochrome P-450- and glutathione-dependent biotransformation of trichloroethylene in humans

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Abstract ***Objectives:*** To investigate in humans the contribution of the cytochrome P-450- and glutathione-dependent biotransformation of trichloroethylene (TRI) under controlled repeated exposure in volunteers, and under occupational conditions. ***Methods:*** Volunteers were exposed to TRI, using repeated 15 min exposures at 50 and 100 ppm. This exposure schedule resulted in internal doses of 1.30 and 2.40 mmol of TRI respectively. Urine samples were collected for a minimum of 45 h. Urine samples were also collected from occupationally exposed workers. The samples were analysed for the known human metabolites of TRI, trichloroethanol (TCE), trichloroacetic acid (TCA) and both regio-isomeric forms of the mercapturic acid *N*-acetyl-*S*-(dichlorovinyl)-L-cysteine (DCV-NAC), and for (dichlorovinyl)-L-cysteine (DCVC). In order to further elucidate the metabolism of TRI in humans, we analysed samples for dichloroacetic acid and for the proposed break-down products of 1,2 and 2,2-dichlorovinyl-L-cysteine after deamination: the *S*-conjugates of 3-mercaptolactic acid, 3-mercaptopyruvic acid and 2-mercaptoacetic acid. ***Results:*** None of the glutathione metabolites was found in urine of volunteers. In workers occupationally exposed to TRI at levels between 0.4 and 21 ppm [8-h time-weighted average (TWA)], levels of DCV-NAC in urine samples collected at the end of the 4th working day and also next morning were below detection limit (0.04 μmol/l). This confirms the findings of Bernauer et al. (1996) that these metabolites are excreted at very low levels in humans. Urinary levels of DCVC and six postulated metabolites of dichlorovinyl-*S*-cysteine conjugates via deamination were also below 0.04 μmol/l, indicating that at most 0.05% of the dose is excreted in the form of these metabolites. These data further strengthen the argument for a very low activity of glutathione-mediated metabolism for chronically exposed workers. ***Conclusions:*** This study gives additional data which indicate that glutathione-mediated metabolism is of minor importance in humans exposed to TRI. In spite of indications to the contrary, significant metabolism of the cysteine conjugate via ß-lyase, which could result in a toxic metabolite, cannot be ruled out completely.

Key words Trichloroethylene · Volunteers · Occupational exposure · Biotransformation · Glutathione · Dichlorovinyl-*S*-cysteine

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Introduction

Trichloroethylene (TRI) has been manufactured on an industrial scale since the beginning of the twentieth century. It is widely used for many different purposes, the main use being metal degreasing (IARC 1995, ECETOC 1994).

The potential carcinogenicity of TRI has been the subject of a number of animal and epidemiological studies (for a recent review see IARC 1995). TRI has

been shown to increase the incidence of lung and liver tumours in the mouse. A slight increase in the number of renal tubule adenocarcinomas was observed in the male rat in three of the seven strains tested.

The epidemiology of TRI is extensive, and includes six occupational cohort studies. These studies covered 20,000 TRI-exposed workers, with often more than 40 years of follow up. Taken together, these studies do not demonstrate a link between exposure to TRI and renal cancer incidence or mortality (Shindell and Ulrich 1985; Axelson et al. 1994 Antilla et al. 1995, Blair et al. 1998; Morgan et al. 1998. Ritz 1999. Boice et al. 1999). No statistically significant elevations in occurrence were seen for renal cell cancer. Henschler et al. (1995) however, link an increase in renal cancer in a group of 169 cardboard workers with exposure to TRI. The explanation given for this finding is the very high exposure to TRI, which the workers would have endured for a long time. Lack of exposure measurements in this retrospective study, do not allow substantiation of this claim (Bloemen and Tomenson 1996. Henschler et al. 1996; Swaen 1996). A small case-control study by Vamvakas et al. (1998), conducted in the same area but on workers from other industries, seems to confirm this suggestion, but questions have been raised about the design of this last study (Green and Lash 1999).

TRI is metabolized by two pathways: oxidation by cytochrome P450, and conjugation with glutathione (Davidson and Beliles 1991, Goeptar et al. 1995). P450-metabolism of TRI leads to the formation of trichloroethanol (TCE) and trichloroacetic acid (TCA) as major metabolites. Minor metabolites formed through this pathway include carbon dioxide, dichloroacetic acid (DCA) and *N*-(hydroxyacetyl)-aminoethanol (ECETOC 1994) (Fig. 1).

A number of potential mechanisms have been postulated for the renal toxicity and carcinogenicity of TRI in male rats: chronic cytotoxicity and cell proliferation, protein droplet nephropathy and activation by the glutathione pathway. Of these, cytotoxicity appears to be a significant factor, as kidney tumours in rats have been observed only in the presence of clear nephrotoxicity (ECETOC 1994). Protein droplet nephropathy was not seen in male rats given high doses of TRI by gavage, daily for 42 days (Green et al. 1990).

The glutathione conjugation pathway has been extensively studied. The TRI-glutathione conjugate is further metabolized by peptidases, yielding a cysteine-conjugate. For TRI the conjugate occurs in two regio-stereo-isomeric forms, *S*-1,2- and *S*-2,2-dichlorovinyl-L-cysteine (DCVC). DCVC may be a substrate for multiple enzymes, among which is cysteine conjugate ß-lyase (Commandeur et al. 1995, Koob and Dekant 1991). The product of this pathway is thought by some to be responsible for the renal cytotoxicity seen in rats. The pathways are represented for the 1,2-isomers in Fig. 2.

The main urinary metabolite resulting from the conjugation by glutathione for most species is the corresponding *N*-acetyl cysteine conjugate. Therefore, it is anticipated that *N*-acetylated DCVC will be the most appropriate metabolite to use to assess the extent of metabolism of TRI by this route. The pathway starting with conjugation with glutathione has been shown to be active for TRI at a very low level in rats and mice (Dekant et al. 1984; Commandeur and Vermeulen 1990), as indicated by the low levels of urinary *N*-acetyl DCVC. Evidence for the existence of this pathway in humans has recently been published (Birner et al. 1993; Bernauer et al. 1996; Brüning et al. 1998).

Bernauer et al. (1996) measured *N*-acetyl-*S*-(dichlorovinyl)-L-cysteine (DCV-NAC) in urine of humans and rats after inhalation of TRI, and compared this with the excretion of TCA and TCE. Their results show excretion of DCV-NAC to be 1,000 to 7,000 times less than that of the metabolites of the oxidative pathway, TCE and TCA. The data from Bernauer et al. (1996) are not in accordance with data from Birner et al. (1993) which indicate a much higher contribution of the glutathione pathway in the metabolism of TRI in humans and rats. Assessment of the exposure conditions was much more elaborate in the Birnauer study, which could account for the discrepancy.

This study investigates the contribution of the glutathione pathway to the total metabolism of TRI in humans. This is done by quantifying both known and

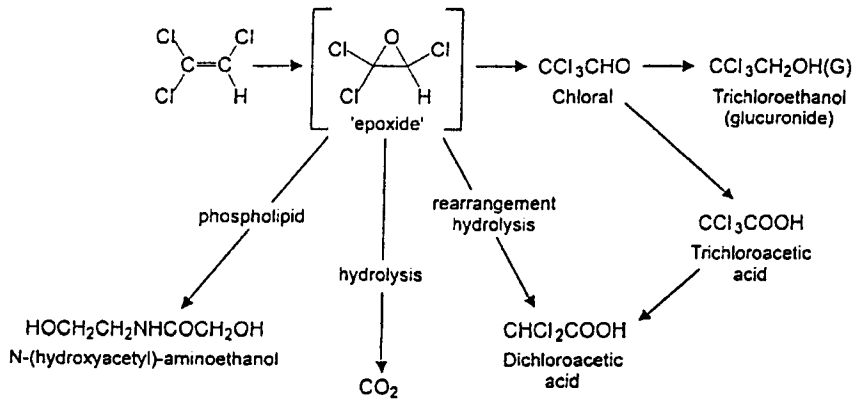

Fig. 1 Cytochrome P-450 metabolic pathway of trichloroethylene

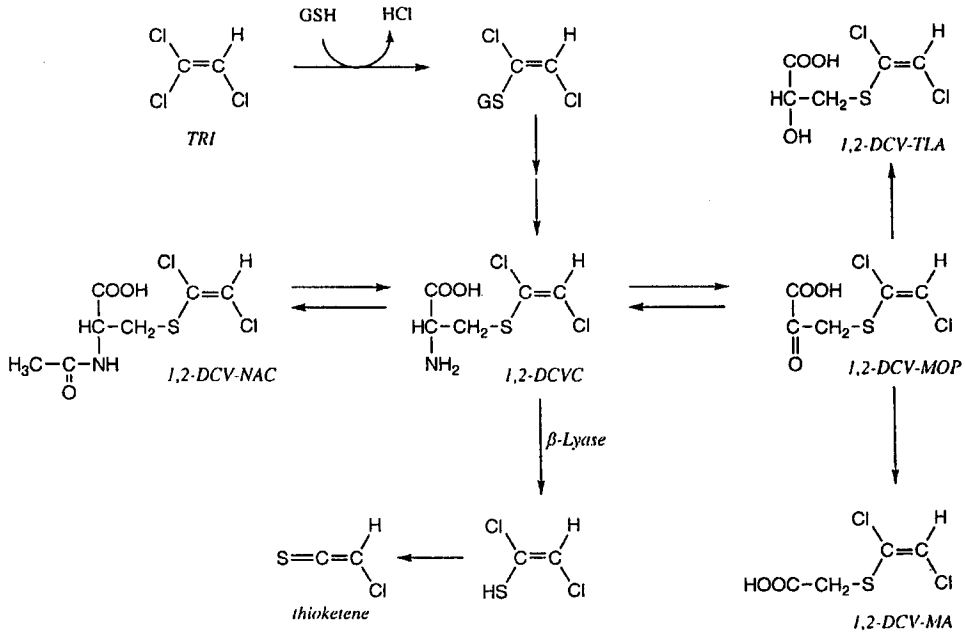

Fig. 2 Glutathione (GSH) metabolic pathway of trichloroethylene; only 1,2 stereo-isomer is shown

suggested end-products of the oxidative and glutathione pathways after controlled, short-term exposure, and after repeated occupational exposure to TRI. The controlled exposure conditions allowed for exact measurement of the uptake of TRI, while the occupational setting gave exposure conditions, which had a longer duration and a potentially higher level of exposure to TRI.

Materials and methods

Controlled exposure

The controlled exposures were carried out at the Coronel Institute, Academic Medical Centre, Amsterdam. Four male adults, aged between 35 and 45, were exposed under three conditions: four times 15 min exposure to 100 ppm, eight times 15 min exposure to 50 ppm and eight times 15 min exposure to 100 ppm. Exposure periods were separated by 10-min exposure-free intervals. This set-up was chosen because the volunteers had to inhale dry air enriched with TRI, which becomes difficult after some time. Therefore a short recovery period was introduced. The maximum exposure levels in the experimental setting were valued against the Dutch occupational exposure standard for TRI, which is 35 ppm (190 mg/m^3) 8-h time-weighted average, with a short-term (15 min) exposure limit of 100 ppm (538 mg/m^3).

The exposure regime was randomly distributed among the volunteers. In order to ensure that TRI and its metabolites were almost totally excreted (urinary TCA half-life ca. 100 h), the interval between exposure for each volunteer was 3 weeks.

Exposure conditions were essentially the same as those described by Monster et al. (1976). The volunteers inhaled air with the required concentration of TRI from a Tedlar bag through a mouthpiece equipped with valves. Total exhaled air was collected in another Tedlar bag, and its volume was measured. The concentration of TRI in inhaled air was measured before and four times during each exposure. The concentration of TRI in exhaled air was measured seven times during each exposure, after collection of alveolar air in glass tubes. The concentration of TRI in the total exhaled air was measured in the Tedlar bag. This enabled exact measurement of the internal dose of TRI.

Urine samples were collected in separate aliquots immediately before, the day after, and the morning following exposure. Urine samples collected during the subsequent 24 h were pooled. Samples were kept at −20 °C.

Prior to inclusion in the study, each volunteer signed a written informed consent. The protocol was reviewed and accepted by the Medical Ethical Committee of the Academic Medical Centre, and the study was therefore performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Occupational exposure

The study involved 11 workers who were exposed to TRI. Spot urine samples were collected at the beginning of the shift, at the end of the fourth day and the fifth day before work. During these four days, passive sampling of air breathed on 3M badges was used to assess exposure to TRI via inhalation. Upon collection, urine samples were divided into fractions, frozen and stored at −20 °C.

Synthesis of reference chemicals

S-(1,2-dichlorovinyl)-L-cysteine, *S*-(2,2-dichlorovinyl)-L-cysteine, *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine and *N*-acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine were prepared as described previously (Commandeur and Vermeulen, 1990).

N-acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine (TCV-NAC) was prepared according to Dekant et al. (1986).

S-(1,2-dichlorovinyl)-3-thiolactic acid (1,2-DCV-TLA) and *S*-(2,2-dichlorovinyl)-3-thiolactic acid (2,2-DCV-TLA) were synthesized from the corresponding cysteine *S*-conjugates according to the procedure described by Kinuta et al. (1994). Eight portions of 250 µl 2 N hydrochloric acid were added to a cold (<4 °C) solution of 100 mg cysteine *S*-conjugate in 2 ml 2 N sodium nitrite. After each addition of hydrochloric acid, the reaction mixture was vortexed for 1 min and left on ice for 2 min. After the last addition of hydrochloric acid, the reaction mixture was left for 1 h at room temperature. The reaction mixture was subsequently acidified to pH 2 and extracted with two portions of 5 ml of ethylacetate. The ethylacetate fractions were combined and evaporated to dryness. The methyl esters were prepared by treatment with ethereal diazomethane, and subsequently analysed by gas chromatography-mass spectrometry (GC-MS), as described below. This procedure

resulted in major products with the expected molecular ions, m/z 230 (with two chlorine isotope patterns), and fragmentation patterns.

S-(1,2-dichlorovinyl)-3-thiolactic acid methyl ester: retention time, 7.30 min; m/z (intensity %, multiplicity, assignment) 230 (13%, 2Cl, $M^{+\cdot}$), 212 (3%, 2Cl, $M^{+\cdot}$–H_2O), 194 (51%, 1Cl, $M^{+\cdot}$–HCl), 177 (39%, 1Cl, $M^{+\cdot}$–Cl–H_2O), 171 (12%, 2Cl, $M^{+\cdot}$–$COOCH_3$), 167 (21%), 169 (13%), 141 (51%, 2Cl, $C_2HCl_2SCH_2^+$), 127 (25%, 2Cl, $C_2HCl_2S^+$), 119 (21%), 107 (21%), 94 (21%), 92 (50%), 79 (50%), 59 (82%), 45 (100%).

S-(2,2-dichlorovinyl)-3-thiolactic acid methyl ester: retention time, 7.45 min; m/z (intensity %, multiplicity, assignment) 230 (43%, 2Cl, $M^{+\cdot}$), 212 (12%, 2Cl, $M^{+\cdot}$–H_2O), 177 (6%, 1Cl, $M^{+\cdot}$–Cl–H_2O), 171 (3%, 2Cl, $M^{+\cdot}$–$COOC\ H_3$), 167 (3%), 141 (100%, 2Cl, $C_2HCl_2SCH_2^+$), 127 (26%, 2Cl, $C_2HCl_2S^+$), 119 (25%), 107 (24%), 94 (9%), 92 (22%), 79 (22%), 59 (41%), 45 (99%).

S-(1,2-dichlorovinyl)-3-mercapto-2-oxopropionic acid (1,2-DCV-MOP) and *S*-(2,2-dichlorovinyl)-3-mercapto-2-oxopropionic acid (2,2-DCV-MOP) were prepared biosynthetically by incubating the corresponding cysteine *S*-conjugates at a concentration of 4 mM in the presence of 1 mg/ml commercial L-amino acid oxidase and 4,000 U/ml catalase. The oxidative deamination reactions were carried out at 37 °C in 50 mM potassium phosphate buffer pH 7.4. After 60 min of incubation, the reaction mixtures were acidified to pH 2 and extracted with two portions of 5 ml of ethylacetate. The combined ethylacetate fractions were combined, evaporated to dryness and treated with ethereal diazomethane. According to GC-MS, the product was methylated at two positions by this procedure, yielding *S*-(dichlorovinyl)-3-mercapto-2-methoxypropenic acid methyl ester, as described previously (Tomisawa et al. 1986). Due to cis- and trans-conformations of the propenic acid moiety, two regio-isomers were found upon GC-MS-analysis.

S-(1,2-dichlorovinyl)-3-mercapto-2-methoxypropenic acid methyl ester.

Isomer 1: retention time, 7.74 min; m/z (intensity %, multiplicity, assignment) 242 (3%, 2Cl, $M^{+\cdot}$), 183 (5%, 2Cl, $M^{+\cdot}$–$COOH_3$), 148 (8%, 1Cl, $M^{+\cdot}$–$COOH_3$–Cl), 135 (10%), 105 (11%), 92 (11%), 85 (11%), 75 (12%), 59 (79%), 45 (100%).

Isomer 2: retention time, 7.70 min. 242 (0.8%, 2Cl, $M^{+\cdot}$), 183 (0.8%, 2Cl, $M^{+\cdot}$–$COOH_3$), 147 (2%, $M^{+\cdot}$–C_2HCl_2), 115 (9%, $M^{+\cdot}$–SC_2HCl_2), 92 (19%), 83 (29%), 79 (15%), 59 (49%), 45 (100%).

S-(2,2-dichlorovinyl)-3-mercapto-2-methoxypropenic acid methyl ester.

Isomer 1: retention time, 7.95 min; m/z (intensity %, multi plicity, assignment) 242 (3%, 2Cl, $M^{+\cdot}$), 183 (4%, 2Cl, $M^{+\cdot}$–$COOH_3$), 148 (6%, 1Cl, $M^{+\cdot}$–$COOH_3$–Cl), 128 (5%), 105 (6%), 92 (5%), 85 (9%), 75 (13%), 59 (82%), 45 (100%).

Isomer 2: retention time, 7.90 min. 242 (1%, 2Cl, $M^{+\cdot}$), 183 (0.5%, 2Cl, $M^{+\cdot}$–$COOH_3$), 141 (2%), 128 (4%), 115 (3%, $M^{+\cdot}$–SC_2HCl_2), 83 (25%), 59 (30%), 45 (100%).

Reference samples of *S*-(1,2-dichlorovinyl)-2-mercaptoacetic acid (1,2-DCV-MA) and *S*-(2,2-dichlorovinyl)-2-mercaptoacetic acid (2,2-DCV-MA) were prepared by treating the corresponding 3-mercapto-2-oxopropionic acid *S*-conjugates with hydrogen peroxide. The 3-mercapto-2-oxopropionic acid *S*-conjugates were prepared by the procedure described above, but in the absence of catalase. After 60 min of oxidative deamination, hydrogen peroxide was added to a final concentration of 1 mM and the reaction mixture was left for another 60 min at room temperature. The reaction mixture (total volume 5 ml) was subsequently acidified to pH 2 and extracted twice with 5 ml portions of ethylacetate. The combined ethylacetate extracts were evaporated and treated with diazomethane dissolved in ether prior to analysis by GC-MS.

S-(1,2-dichlorovinyl)-2-mercaptoacetic acid methyl ester: retention time, 5.78 min; m/z (intensity %, multiplicity, assignment) 200 (51%, 2Cl, $M^{+\cdot}$), 165 (82%, 1Cl, $M^{+\cdot}$–Cl), 141 (82%, 2Cl, $M^{+\cdot}$–$COOCH_3$), 127 (13%, 2Cl, $C_2HCl_2S^+$), 94 (21%), 92 (50%), 79 (100%), 59 (50%), 57 (36%), 45 (100%).

S-(2,2-dichlorovinyl)-2-mercaptoacetic acid methyl ester: retention time, 5.94 min; m/z (intensity %, multiplicity, assignment) 200 (95%, 2Cl, $M^{+\cdot}$), 165 (91%, 1Cl, $M^{+\cdot}$–Cl), 141 (92%, 2Cl, $M^{+\cdot}$–$COOCH_3$), 127 (10%, 2Cl, $C_2HCl_2S^+$), 94 (14%), 92 (51%), 79 (100%), 59 (55%), 57 (41%), 45 (100%).

Identification and quantification of glutathione-derived metabolites of TRI in human urine

Method A (analysis of mercapturic acids, 3-thiolactic acid S-conjugates, 2-mercaptoacetic acid S-conjugates and 3-mercapto-2-oxopropionic acid S-conjugates)

1.5 µg *N*-acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine (TCV-NAC) was added to 3 ml of human urine as an internal standard. The urine samples were acidified with 200 µl 2 N hydrochloric acid, resulting in a pH of 1–2. Two volumes of ethylacetate were used for extraction by vortex-mixing for 1 min. Thereafter, centrifugation took place at 4,000*g* for 5 min. The combined ethylacetate fractions were evaporated under a gentle nitrogen stream in a water bath at 37 °C. The residues were dissolved in 500 µl of methanol, and 1 ml diazomethane dissolved in ether was added, and the mixture allowed to react for 1 h at room temperature. After evaporation of the solvents and excess diazomethane, the final residue was dissolved in 250 µl ethylacetate and analysed by GC-MS.

Calibration curves of the mercapturic acids were prepared by dissolving 1,2-DCV-NAC and 2,2-DCV-NAC in human urine at concentrations ranging from 10 ng/ml to 1,000 ng/ml. To 3 ml samples of these solutions, 1.5 µg TCV-NAC was added as an internal standard. The subsequent isolation and derivatization procedures were executed as described above.

Method B (analysis of cysteine S-conjugates)

For identification of cysteine *S*-conjugates, urine samples were treated with acetic anhydride under alkaline conditions prior to the acid extraction procedure. During the procedure, the temperature of the samples was always kept at < 4 °C in order to avoid alkaline degradation of thioether metabolites.

1.5 µg TCV-NAC was added to 3 ml of human urine as an internal standard. 1 ml 4 N sodium hydroxide was added to 3 ml human urine. 25 µl fractions of acetic anhydride were added to this mixture while vortexing. After each addition of acetic anhydride the mixture was left on ice for 2 min. When the pH of the reaction mixture was decreased to pH 6–7, the reaction mixture was left for 20 min at room temperature. The samples were subsequently acidified to pH 1–2 and extracted twice with two volumes of ethylacetate by vortex-mixing for 1 min. After centrifugation (2,500*g* for 5 min), the combined ethylacetate fractions were evaporated under a gentle nitrogen stream in a water bath at 37 °C. The residues were dissolved in 500 µl of methanol, and 1 ml ethereal diazomethane was added, which was allowed to react for 1 h at room temperature. After evaporation of the solvents and excess diazomethane, the final residue was dissolved in 250 µl ethyl acetate and analysed by GC-MS.

Instrumental analysis

All GC-MS analyses were carried out on a Hewlett Packard 5890/MSD system. A CP Sil-SE 30 capillary column (25 m, 0.22 mm i.d.) obtained from Chrompack Ned. BV was used. The operation temperatures were 280 °C (split injector) and 280 °C (ion source). Electron impact ionization (electron energy of 70 eV) was used. The carrier gas was helium, at a flow rate of approximately 3 ml/min, and the column head pressure was 80 kPa. The column temperature was programmed from 60 °C (2.5 min) to 280 °C at 20 °C /min. Using these conditions the retention times of the methyl esters of 1,2-DCV-NAC and 2,2-DCV-NAC were 9.12 and 9.20 min, respectively. Retention time of the methyl ester of the internal standard TCV-NAC was 9.79 min.

The presence of glutathione-derived metabolites and internal standard in urine extracts was analysed for by selected ion monitoring by programming the following ions:

1,2-DCV-NAC and 2,2-DCV-NAC: m/z 177, 212 and 214
1,2-DCV-TLA and 2,2-DCV-TLA: m/z 141, 194 and 230
1,2-DCV-MOP and 2,2-DCV-MOP: m/z 242 and 244
1,2-DCV-MA and 2,2-DCV-MA: m/z 200 and 202
TCV-NAC (internal standard): m/z 246 and 248

The detection limit for the mercapturic acids and cysteine conjugates in urine was 0.04 μmol/l (signal to noise ratio ~5). The detection limits for the other sulphur-containing metabolites could not be detected accurately because of the presence of impurities in the reference materials. However, when concentrations of stock solutions were standardized according to responses on flame ionization detector (FID) and flame photometric detector (FPD) detectors, detection limits of 0.1 μmol/l were obtained using GC-MS procedures.

TCE glucuronide in urine was hydrolysed by pretreatment with ß-glucuronidase. TCE was extracted in toluene. After acidification of the sample, trichloroacetic acid was extracted in toluene and methylated by adding borotrifluoride-methanol. TCE and methylated TCA were analysed by gas-chromatography and electron capture detection. The detection limit was 0.01 mmol/l for both metabolites (Monster et al. 1976).

Dichloracetic acid analysed in the same way as TCA. The detection limit was 0.01 mmol/l.

Results

Controlled exposure study

In the controlled exposure study, quantification of the internal dose was obtained by comparison of the difference in levels of TRI between inhaled and exhaled air and measurement of the ventilation volume. Two different exposure regimes with the same 8-h time-weighted exposure average of 12.5 ppm were applied: four times 15 min exposure to 100 ppm and eight times 15 min exposure to 50 ppm. Both resulted in virtually the same uptake, 1.30 vs 1.31 mmol TRI, respectively, as an average for the four volunteers (Table 1). The third exposure regime, eight times 15 min exposure to 100 ppm (25 ppm 8-h TWA) gave an average internal dose of 2.40 mmol TRI. The measurements of uptake of inhaled TRI were only slightly higher than those predicted from the data from Monster et al. (1976) which would be 1.06 and 2.13 mmol TRI.

TCE, excreted in urine during the two days after exposure, constituted on average 20% of the inhaled dose of TRI (range 13–27%, Table 1). No difference in percentage TCE was seen between the three different exposure regimes although there was a tendency for higher TCE excretion at the highest exposure level. There was a consistent difference in retention of inhaled TRI between persons but the variation in excretion of metabolites of TRI as a percentage of inhaled dose was not related to these differences.

The TCA recovered in the urine samples collected for 46 h, as percentage of inhaled dose, was 3.3% (range 1.0–7.4%). Recovery of TCA was so low because of the long half-life of TCA [(100 h, Monster et al. (1976)].

Despite several efforts, none of the metabolites of the glutathione conjugate of TRI could be detected in any of the urine samples collected during the first day after exposure or in the pooled samples from the second day after exposure. Because detection limits for each of these metabolites was 0.04 μmol/l, it can be calculated that glutathione conjugation, as expressed in the urinary excretion of the ten conjugates monitored, represents less than 0.05% of the dose (the highest dose of 2.30 mmol TRI resulted in less than 10 × 0.04 μmol/l in the daily excretion of 1.5 l of urine). When ignoring possible excretion of the other eight metabolites, the two isomers of DCV-NAC can at most represent 0.01% of the dose of TRI.

Dichloroacetic acid could also not be found in urine samples, while the detection limit was 0.01 mmol/l. This finding suggests that in the oxidative metabolic pathway, formation of DCA from trichloroethylene-epoxide or from TCA is either not occurring or is occurring at a very low level in humans at these exposure levels.

Occupational exposure study

Inhalation exposure to TRI for 11 workers involved in the application of carbon black dissolved in TRI was assessed during four consecutive days by passive air sampling. Exposure to TRI in this situation was continuous for the whole working day although at varying levels. Daily exposures ranged from 0.4 to 21 ppm (8-h TWA). Average exposure during the four-day study

Table 1 Volunteer study: uptake of trichloroethylene and excretion of trichloroethanol (*TCE*) and trichloroacetic acid (*TCA*)

Volunteer	4 × 100 ppm				8 × 50 ppm				8 × 100 ppm			
	Dose (mmol)	Sampling time of urine (hrs)	TCE (% dose)	TCA (% dose)	Dose (mmol)	Sampling time of urine (hrs)	TCE (% dose)	TCA (% dose)	Dose (mmol)	Sampling time of urine (hrs)	TCE (% dose)	TCA (% dose)
1	1.21	45	20	1.5	1.30	48	16	6.3	1.90	48	27	4.2
2	1.30	45	21	3.3	1.40	48	17	2.2	2.90	46	21	7.4
3	1.50	46	14	1.0	1.30	52	22	2.8	2.50	44	22	2.0
4	1.20	45	13	2.1	1.30	46	12	2.3	2.30	46	20	4.0
Average	1.30	–	17	1.97	1.33	–	17	2.4	2.4	–	23	4.4
SD	0.14	–	4.1	0.99	0.05	–	4.1	0.27	0.42	–	3.1	2.23

period for individual workers ranged from 1.4 to 14.1 ppm (Table 2).

Spot urine samples were collected at the beginning of the shift, at the end of the fourth day, and on the following morning before work. Levels of TCE and TCA in samples collected at the end of the fourth day ranged from respectively 0.03–0.30 mmol/l and 0.02–0.25 mmol/l. These levels are consistent with the exposure levels found by personal monitoring when applying the levels suggested by the German MAC and BAT-list, where 50 ppm is regarded as equivalent to 0.61 mmol/l TCA in end-of-shift urine samples (Deutsche Forschungsgemeinschaft 1996). DCA levels were below detection limit in the samples.

The various potential products of the glutathione pathway were not detected.

Six urine samples from one of the volunteers, collected during the first day after the highest exposure regime, and two samples from one of the workers (F in Table 2) were also analysed for the two stereo-isomers of DCV-NAC by a second laboratory, where a lower limit of detection was available (0.004 μmol/l). The method used is described by Bernauer et al (1996). No DCV-NAC was detected in the samples from the controlled exposure. In the samples of the occupational exposure situation DCV-NAC was found. End-of-shift level of 1,2 DCV-NAC was 0.004 μmol/l, and 0.057 μmol/l 2,2 DCV-NAC. Next-morning levels were respectively 0.003 and 0.070 μmol/l. These levels are in the range of the detection limit of our method and these results therefore confirm our findings.

Discussion

In this study the uptake of TRI was investigated under controlled conditions at and above relevant occupational exposure levels. Furthermore, the excretion of identified and postulated end-products of the metabolism of TRI in humans was studied under short-term, controlled and long-term, occupational exposure conditions. Urinary levels of TCE, TCA, and DCA were measured as end-products of oxidative biotransformation of TRI. The contribution of glutathione conjugation in the metabolism of TRI was studied by measuring urinary levels of 1,2 and 2,2-DCVC, 1,2 and 2,2-DCV-NAC and the products of postulated metabolism of cysteine conjugates via transamination (1,2-DCV-MOP and 2,2-DCV-MOP) and subsequent decarboxylation (1,2-DCV-MA and 2,2-DCV-MA) or reduction (1,2-DCV-TLA and 2,2-DCV-TLA).

Table 2 Personal monitoring results from occupationally exposed workers

Volunteer	Exposure level (ppm, 8-h time-weighted average)					
	Day 1	Day 2	Day 3	Day 4	Average	SD
A	1.3	1.9	3.2	2.4	2.2	0.8
B	4.2	3.9	2.3	1.8	3.1	1.2
C	14.3	21.0	14.0	4.4	14.0	6.8
D	0.4	1.9	8.3	1.1	2.9	3.6
E	1.4	0.9	–	1.8	1.4	0.5
F	14.9	10.6	11.9	19.3	14.2	3.9
G	4.8	2.9	2.6	3.9	3.5	1.0
H	3.4	8.8	4.4	6.3	5.7	2.4
I	4.4	1.2	1.4	3.8	2.7	1.6
J	15.0	9.2	3.5	7.5	8.8	4.8
K	3.9	6.8	3.2	3.5	4.4	1.7

In spite of the very low detection limit for the ten end-products of biotransformation of the glutathione conjugate of TRI, we could not detect these metabolites in samples from controlled exposure conditions or occupationally exposed. This indicates that not only the formation of mercapturic acids but also that of other postulated metabolites of the glutathione conjugate of TRI plays a very minor role in the metabolism of TRI in both exposure situations.

Under conditions of controlled exposure to TRI at exposure levels of up to 100 ppm for 8 h, the ratio between excretion of TCE + TCA and the mercapturic acids 1,2 and 2,2 DCV-NAC exceeds 2,300.

Recently Bernauer et al. (1996) published data on DCV-NAC which indicate a ratio of TCE+TCA vs DCV-NAC of between 1,000 and 7,000 in rats and humans, which is well in agreement with our findings in humans.

Brüning et al. (1998) report urinary levels of DCV-NAC of up to 1.25 μmol/g creatinine in a case of acute poisoning from the drinking of TRI. The toxic effects of the very high dose and of the subsequent very intensive treatment, so compromised the patient that it is difficult to interpret these results other than in a qualitative way.

The available data at this moment do not with certainty exclude the possibility of yet another pathway in the glutathione-mediated metabolism playing a significant role. Bioactivation of the dichlorovinyl-cysteine intermediate by β-lyase results in a reactive sulphur-containing metabolite in some species. Until the reactive metabolite or the covalent binding product of this reactive metabolite has been quantified, some uncertainty still exists on this aspect. The low activity of β-lyase in human liver in vitro, compared with rats, does suggest a lower level of activity of this pathway in humans (Green et al. 1997).

It has been argued that high exposures to TRI could result in non-linear biotransformation responses. Our data show that the uptake of TRI via inhalation was linear up to 100 ppm and independent of the exposure scenarios used. Excretion of the end-products of the oxidative pathway was also linear up to 100 ppm. At exposure levels of 160 ppm humans and rats showed a levelling of the amount of urinary DCV-NAC excreted (Bernauer 1996). This suggests a decreased relative importance of the glutathione pathway at higher exposure levels.

Overall, the available data give strong, although not conclusive, evidence that glutathione-mediated metabo-

lism of TRI is a very minor pathway in humans. The study design does not allow for direct conclusions regarding carcinogenicity. In spite of indications to the contrary, significant metabolism of the cysteine conjugate via β-lyase, which could result in a toxic metabolite, cannot be ruled out completely. Our data suggest that under chronic exposure conditions the formation of *N*-2,2-DCVC may be favoured in comparison with the more toxic *N*-1,2-DCVC.

Studies on enzyme activities and products of covalent binding will be helpful in providing further insight into the mechanism of renal cancer generation in male rats of some strains, and the relevance of these findings for humans.

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