From:	Barajas-Ochoa, Esther@OEHHA
To:	Robinson, Michelle@OEHHA
Subject:	FW: RESPONSE TO REQUEST FOR RELEVANT INFORMATION ON THE CARCINOGENIC HAZARDS OF DIAMINOTOLUENES - PART 1
Date:	Friday, September 04, 2015 12:27:54 PM
Attachments:	2014-12-22 Ltr re Request for Data on Diaminotoluenes.pdf 2014-12-22 Melikyan Declaration.pdf Exh A - Melikyan CV.pdf Exh B - 1988 - DAT (mixed) basis document CalEPA OEHHA.pdf

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From: Peter T. Sato [mailto:peter@yeroushalmi.com]
Sent: Monday, December 22, 2014 2:50 PM
To: P65Public Comments
Cc: 'Reuben Yeroushalmi'
Subject: RESPONSE TO REQUEST FOR RELEVANT INFORMATION ON THE CARCINOGENIC HAZARDS OF DIAMINOTOLUENES - PART 1

Please refer to the attached. This submission will include subsequent emails due to the submission's size.

Very truly yours,

Peter T. Sato

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December 22, 2014

VIA E-MAIL ONLY [P65Public.comments@oehha.ca.gov]
The Carcinogen Identification Committee
Office of Environmental Health Hazard Assessment
1001 I Street
Sacramento, California 95814

Re: ***REQUEST FOR RELEVANT INFORMATION ON THE CARCINOGENIC HAZARDS OF DIAMINOTOLUENES.***

Dear Mesdames and/or Sirs:

Enclosed you will find Plaintiff Consumer Advocacy Group, Inc.'s ("CAG") response to the Office of Environmental Health Hazard Assessment's ("OEHHA") Request for Relevant Information on the Carcinogenic Hazards of Diaminotoluenes ("Request") and in turn, the response to Defendant, Big Lots Stores, Inc.'s ("Defendant") October 21, 2014 petition for reconsideration of listing for diaminotoluene (mixed).

Attached is the declaration of Dr. Gagik Melikyan ("Melikyan Declaration") which highlights and discusses information on toxicity of Diaminotoluenes as a group.

In addition, CAG responds to Big Lot's petition as follows:

A. Big Lot's Argument that the EPA Has "Not Formally" Identified Diaminotoluene (Mixed) as Causing Cancer is Based on the Wrong Listing Document

A chemical is "formally identified" by an authoritative body when the lead agency determines that:

> (1) the chemical has been included on a list of chemicals causing cancer or reproductive toxicity issued by the authoritative body; or is the subject of a report which is published by the authoritative body and which concludes that the chemical causes cancer or reproductive toxicity; **or** has otherwise been identified as causing cancer or reproductive toxicity by the authoritative body in a document that indicates that such identification is a final action; **and**
> (2) the list, report, or document specifically and accurately identifies the chemical, and has been:

(A) Reviewed by an advisory committee in a public meeting, if a public meeting is required, **or**
(B) Made subject to public review and comment prior to its issuance, **or**
(C) Published by the authoritative body in a publication, such as, but not limited to, the federal register for an authoritative body which is a federal agency, **or**
(D) Signed, where required, by the chief administrative officer of the authoritative body or a designee, **or**
(E) Adopted as a final rule by the authoritative body, **or**
(F) Otherwise set forth in an official document utilized by the authoritative body for regulatory purposes.[1]

Big Lot's argument relies on the false premise that the listing of Diaminotoluene (Mixed) was solely based upon the document, "EPA, 1986, External Review Draft: Methodology for Evaluating Potential Carcinogenicity to Support Reportable Quantity Adjustments Pursuant to CERCLA Section 102, Office of Health and Environmental Assessment, OHEA-C-073" ("*EPA 1986 Document*") However, the listing was also based on "Evaluation of the Potential Carcinogenicity of Diaminotoluene (Mixed) (95-80-7). In Support of Reportable Quantity Adjustments Pursuant to CERCLA Section 102, Carcinogen Assessment Group, Office of Health and Environmental Assessment Washington, DC, 20460 EPA/600/8-91/103 June 1988" (*EPA 1988 Document*").[2] Big Lot's petition does not even address the *EPA 1988 Document*.

The EPA 1988 Document satisfies the criteria under 27 *CCR* § 25306(d)(1) because it shows that Diaminotoluene (Mixed) is the "subject of a report which is published by the authoritative body and which concludes that the chemical causes cancer or reproductive toxicity" and "has otherwise been identified as causing cancer or reproductive toxicity by the authoritative body (EPA) in a document that indicates that such identification is a final action."

The procedures under 27 *CCR* § 25306(d)(2) do not require that every report must be made subject to public review and comment. Such report can also be "Published by the authoritative body in a publication, such as, but not limited to, the federal register for an authoritative body which is a federal agency" (27 *CCR* § 25306(d)(2)(B), or "Otherwise set forth in an official document utilized by the authoritative body for regulatory purposes" (27 *CCR* § 25306(d)(2)(F). Here, the *1988 EPA Document* was published. See *Id.* at p. 11. It was also published in the National Technical Information Service ("NTIS"), as it as a NTIS reference number "PB93-185270." It also was formally adopted as an official EPA document and given an EPA reference number "EPA/600/8-91/103." *Id.* at p.1-2.

Hence, Diaminotoluene (Mixed) has been formally identified.

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[1] 27 Cal. Code Reg. § 25306
[2] http://oehha.ca.gov/prop65/CRNR_notices/chemicals_reconsideration/102314Diaminotoluene.html

B. The Diaminotoluene (Mixed) Listing is Not Vague and Ambiguous Because the Listing Document Clearly Indicates it Applies to All Isomers

Further, there is no ambiguity considering that the *EPA 1988 Document* states that "diaminotoluene is assigned a 'MEDIUM' hazard ranking for the purposes of RQ adjustment, which assignment is applicable to all isomers of diaminotoluene." *Id.* at p. v (Abstract). Further, as noted in Dr. Melikyan's Declaration, there are only 6 possible isomers of diaminotoluene, so the listing is clear as to which chemicals it refers to. *Melikyan Declaration*, p.6.

C. The Diaminotoluene (Mixed) Listing Does not Violate Due Process

In its Petition, Big Lots relies on *Dow Chemical, USA v. Consumer Product Safety Commission* (1978) 459 F. Supp. 378, as standing for the proposition that due process requires that the public have notice and the right to comment at the time the government finds that a health hazard is posed by a chemical. Petition at p.5.

However, *Dow Chemical* does not stand for this; it had nothing to do with due process per se, but rather compliance the Federal Administrative Procedures Act ("APA"). There, the court hold that Consumer Product Safety Commission violated the APA when it listed a chemical without giving notice of the rule making in the Federal Register or soliciting comments as required by the APA. It was about failure to comply with the APA. Conversely here, as Big Lots admits, Proposition 65 contains a provision which provides that the listing of chemicals is exempted from the California Administrative Procedures Act. See *HSC* § 25249.8(e). Further, before listing Diaminotoluene (Mixed), OEHHA gave a formal "notice of intent to list chemicals" under 27 *CCR* § 25306(i). See, Petition, Exhibit A. Finally, as indicated above, the 1988 EPA Document satisfies all criteria for listing Diaminotoluene (Mixed) under the Authoritative Body mechanism. See 27 *CCR* § 25306.

Very truly yours,

YEROUSHALMI & YEROUSHALMI

Peter T. Sato

Attachment(s)[3]

[3] Some of the references in Dr. Melikyan's declaration will be forwarded via separate emails.

DECLARATION of PROFESSOR GAGIK MELIKYAN

pertaining to BIG LOTS STORES' petition to reconsider the Proposition 65 listing of Diaminotoluene (Mixed) as a carcinogen pursuant to the Authoritative Body (AB) mechanism

I, Dr. Gagik Melikyan, declare as follows from my personal knowledge. If called upon, I could and would testify competently as follows:

Part I. Introduction

1. I am giving this declaration as an expert in the fields of organic, organometallic, medicinal, enzymatic, computational, hormonal, and food chemistries, cancer drug development, endocrine disruptors, environmental toxicants, consumer product chemistry, human health risk and exposure assessment, as well as in application of these subjects to litigation cases related to California's Safe Drinking Water and Toxic Enforcement Act of 1986 [1] ("Proposition 65") and pertinent state and federal regulations.

2. I am a Professor of Chemistry in the Department of Chemistry and Biochemistry at California State University Northridge with 40 years of professional experience conducting cutting-edge interdisciplinary research. My original contribution to science has culminated in numerous research papers, reviews, and book chapters (83), including eight invited reviews, as well as presentations (120) at national and international conferences, scientific gatherings, and public appearances (*Exhibit A*). The said contribution was recognized by awarding me the prestigious *Outstanding Faculty Award* and *Jerome Richfield Scholar Award*, the highest distinctions given by the university in recognition of excellence in faculty research. The work emanated from my laboratory was widely covered in print media, and through radio and TV outlets.

3. I am also the author of the award-winning book titled "*Guilty Until Proven Innocent: Antioxidants, Foods, Supplements, and Cosmetics*" (2009, 368pp; www.csun.edu/gmelikyan). In laymen terms, the book explains why chemical compounds in household items, foods, supplements, cosmetics, plasticware, jewelry, and other consumer products can cause irreparable damage to human health by being carcinogens, mutagens, reproductive toxins, or endocrine disruptors. I am also an editor of on-line educational programs (newsletters, youtube videos) that bring the latest news on consumer products and related health issues to the general public.

4. Over the last decade, as an expert and consultant, I have provided litigation support to consumer protection groups, served as an expert witness in toxic consumer product torts (cosmetics; foods; electronics; petrochemicals), supported non-profit organizations in the "failure to warn" litigations and the state actions initiated under the California Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65), petitioned government agencies on listed carcinogens and reproductive toxins, provided consulting services to the cosmetic industry on cosmetic formulations, rendered consulting services on environmental pollution related to irresponsible mining practices and environmental product liability cases, provided technical guidance on sampling protocols for environmental pollutants, prepared expert declarations, provided trial depositions, developed wiping protocol for phthalates, conducted human exposure and health risk analysis, evaluated interactions between commercial products and the consumer population, and quantified the exposures to listed chemicals from materials in dispute according to NSRL and MADL standards.

5. I have a detailed knowledge of California's Safe Drinking Water and Toxic Enforcement Act of 1986 ("Proposition 65") [1] through my involvement with the Consumer Advocacy Group ("CAG"), Inc., as a consultant and an expert. I have an in-depth understanding of the Proposition 65 mission, societal ramifications, public health implications, major provisions, warning requirements to potential exposure to listed chemicals, basis for listing chemicals as carcinogens or reproductive toxins, warning

obligations for businesses, and legal consequences for failure to warn. Since 2009, I have been providing scientific backing to a number of litigations involving consumer products, such as foods, cosmetics, household items, and technical gadgets. The types of activities included evaluation of rebuttal files submitted by businesses in response to CAG-initiated lawsuits alleging violations of the Proposition 65 requirement to warn, assessment of exposure to listed chemicals from consumer products in dispute, providing depositions, and testifying as a court expert witness.

6. I have carefully reviewed the petition by Big Lots Stores, Inc. ("Big Lots") to reconsider the Proposition 65 listing of diaminotoluene (mixed) as a carcinogen [2], as well as the declaration provided by Dr. Chris Mackay in support of said petition ([2]; Exhibit A). I have also reviewed the Proposition 65 document that form the basis for listing diaminotoluene (DAT) as a carcinogen ([3]; *Exhibit B*), as well as a large number of research papers related to the toxicity of isomeric DATs, and their salts. Overall, I have a broad and extensive personal knowledge of the matters relating to the pending litigation case, and I can and I will competently testify thereto.

Part II. Expert Opinion on Defendant's Declaration

7. The declaration provided by Dr. Chris Mackey (from now-on "Declaration") is reviewed and evaluated by me. In this declaration, the consecutive numbering of select entries in the defendant expert's Declaration - all italicized - is retained in order to facilitate the identification of the provisions in question.

8. ***Declaration, entry 7****. I have reviewed the Proposition 65 list of chemicals; the Notice of Intent to List dated November 1, 1989 (Exhibit A); the technical documents that formed the bases for California to include diaminotoluene (mixed) on the Proposition 65 list; and other materials I received from the Office of Environmental Health Hazard Assessment pursuant to a California Public Records Act Request. The EPA document used as the technical basis of the listing is not an examination of the carcinogenic properties of any chemical or group of*

chemicals, but rather a guidance on the general approach proposed by EPA for examining chemicals for carcinogenetic properties.

Rebuttal. It is not true that "The EPA document used … is not an examination of the carcinogenic properties … , but rather a guidance … ." The document that formed the basis for the listing is the one published by the EPA in 1988, and titled "Evaluation of the Potential Carcinogenicity of Diaminotoluene (Mixed) (95-80-7)," Carcinogen Assessment Group, Office of Health and Environmental Assessment, U. S. Environmental Protection Agency (EPA; 600/8-91/103); June 1988" ([3]; *Exhibit B*). Entries 9 and 10 of this declaration (below) provide more details with respect to the EPA document that, in fact, formed the basis for the Proposition 65 listing.

9. ***Declaration, entry 8****. The Proposition 65 Notice of Intent to List diaminotoluene (mixed) was issued pursuant to the authoritative body mechanism cited an EPA determination as the bases for the listing, The EPA determination used, Methodology for evaluating potential carcinogenicity in support of reportable quantity adjustments pursuant to CERCLA section 102, is an external review draft and clearly stated on the report title page (Exhibit C; USEPA. 1986. Methodology for evaluating potential carcinogenicity in support of reportable quantity adjustments pursuant to CERCLA section 102. External review draft. Office of Research and Development, Washington DC)*

DRAFT: DO NOT QUOTE OR CITE

This means that EPA had disavowed the technical merit of the document in the form used by California as the authoritative body finding to add diaminotoluene (mixed) on the Proposition 65 list.[3] Since EPA does not recognize the document as a competent technical analysis, it appears that California should not have used this document as an EPA determination for purposes of authoritative body listing of diaminotoluene (mixed). A copy of the title page of the technical document cited as the basis for the Proposition 65 listing of diaminotoluene (mixed) is provided as Exhibit 3.

Rebuttal. The defendant's expert is using the wrong document to criticize the inclusion of "Diaminotoluene (mixed)" in Proposition 65. It is correct that the EPA first published a draft, in 1986, which is discussed in the defendant expert's Declaration. But, it is incorrect that the Proposition 65 listing of "Diaminotoluene (mixed)" is based on the EPA *draft* document. In fact, in 1988, the final document was published titled "Evaluation of the Potential Carcinogenicity of Diaminotoluene (Mixed) (95-80-7)," Carcinogen Assessment Group, Office of Health and Environmental Assessment, U. S. Environmental Protection Agency (EPA; 600/8-91/103); June 1988 ([3]; *Exhibit* B). The said document formed the basis for inclusion of ***all diaminotoluene isomers*** into Proposition 65, as a single listing, without specifying pertinent CAS numbers, as is the case for other "single listings" that include several substances. And, contrary to the statements made in the expert's Declaration, the final document is not the "Methodology for Evaluating …" but the actual "Evaluation of the Potential Carcinogenicity … ." Entry 10 of this declaration (below) provides more details with respect to the EPA document that, in fact, formed the basis for the Proposition 65 listing. It should also be mentioned that this document is categorized by the EPA, not as a **DRAFT**, but as a **FINAL** document dated June 1988.

10. ***Declaration, entry 9****. The Proposition 65 listing of diaminotoluene (mixed) is unclear and confusing. In chemical nomenclature diaminotoluene is an imprecise term that could, for instance, mean any one of ten different and unique congener substances with the same molecular formula and contain two amine moieties on a toluene structural base. The qualifier "(mixed)" is not a term of chemistry. If it is to be taken at its classical definition, we assume that it referrers to an identifiable entity composed on more than one constituent. The problem is that there is nothing in the State's Proposition 65 listing that defines what the composition of this mixture could be. For instance, it could be more than one congeners of diaminotoluene. Alternately, it could refer to a specific congener of diaminotoluene (e.g. 2,4-diaminotoluene) but refer to a mixture of 2,4-diaminotoluene salts (chlorides, sulfates, phosphates, carbonates, etc) since all diaminotoluene congeners are weak bases. It is impossible to tell since the Proposition 65 listing does not include a CAS number.*

Rebuttal. It is puzzling to me how diaminotoluene, a derivative of toluene with two amino groups attached to the aromatic ring, can have "ten different and unique congener substances" as stated by Dr. Chris Mackay in the Declaration ([2], Exhibit A: p.3, line 8; also shown above). The number of positional isomers theoretically possible is only 6, not 10. And these are isomeric 2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-diaminotoluenes, each with a specific CAS number. Except for 2,4-diaminotoluene, a known carcinogen, the other five isomeric compounds are included in the latest "Request for Relevant Information on the Carcinogenic Hazards of Diaminotoluenes" published by the Office of Environmental Health Hazard Assessment (OEHHA) on October 23, 2014 [4].

The Proposition 65 listing of diaminotoluene (mixed) is not " … unclear and confusing …" as stated by Dr. Chris Mackay in the Declaration ([2], Exhibit A: p.3, line 6; also shown above). The exact interpretation can be easily found in the basis document that served as a scientific justification for including isomeric DATs into the Proposition 65 List of Chemicals. The document in question is titled "Evaluation of the Potential Carcinogenicity of Diaminotoluene (Mixed) (95-80-7)" prepared, as a *final document*, by the Carcinogen Assessment Group, Office of Health and Environmental Assessment under U. S. Environmental Protection Agency (EPA; 600/8-91/103) in June 1988 ([3]; *Exhibit B*). The CAS number used in the title (95-80-7) refers to 2,4-diaminotoluene, a key compound among DAT isomers with established carcinogenicity in animals. At the same time, the title also contains the word "mixed" in parenthesis to include other isomers of DAT. To avoid any confusion, or misreading, the document explicitly clarifies the true meaning of the word (mixed) on the "Technical Report Data" page:

> "Evidence on potential carcinogenicity from animal studies is "Sufficient," … . This evidence is based on the carcinogenic properties of the isomer 2,4-diaminotoluene … which assignment is **applicable to all isomers of diaminotoluene**."

The same clarification is repeated in the Abstract of the same EPA document:

> "Diaminotoluene is a probable human carcinogen, classified as Group 2 under the EPA Guidelines for Carcinogen Risk Assessment (U. S. EPA, 1986). … assignment is **applicable to all isomers of diaminotoluene**."

The same clarification is repeated – again – in the same EPA document, under section 3.0 titled "Hazard Ranking":

> "This hazard ranking, which is based on the isomer 2,4-diaminotoluene, **is applicable to all isomers of diaminotoluene.**"

Further clarifications are readily available - upon request – from the OEHHA Cal EPA professionals involved with Proposition 65. On January 24, 2013, at 12:50pm, I had a detailed telephone conversation with Dr. Marta Sandy, Head of the Carcinogenicity Department, and I was told that the word "(mixed)" in the Proposition 65 Listing pertaining to DAT means ***all DAT isomers, any combination thereof, and in any relative amounts.*** It means that any mixture of DAT isomers containing either one, or two, or five isomers, is considered carcinogenic, and thus a listed chemical.

I also strongly disagree with Dr. Chris Mackay's statement regarding a lack of specificity with respect to the "Diaminotoluene(mixed)" listing ([2], Exhibit A: p.3, lines 11/12: *The problem is that there is nothing in the State's Proposition 65 listing that defines what the composition of this mixture could be*). In two paragraphs above, the explanation is already given what the basis document is, and what exactly the terminology used means. In addition, it should be mentioned that the only reason why the pertinent CAS numbers are not shown in the Proposition 65 listing for the "Diaminotoluene (mixed)" entry is because the State Agency was supposed to show all six CAS numbers for all six positional isomers of diaminotoluene. Or, at least, five CAS numbers since 2,4-diaminotoluene (CAS 95-80-7) is already shown in the line above. Understandably, because of the space constraints, the State Agency could not allocate five lines in Proposition 65 Listing for diaminotoluene

isomers.

It should be emphasized that the DAT (mixed) case is not the only one where the Proposition 65 Listing does not contain CAS numbers [1]. These are cases when the entries pertain to more than one compound in the same group, or the same class, and it is completely justified, and logical, not to add CAS numbers to *each* representative of the group, or class. Had that been the case, then the volume of the Proposition 65 Listing would have increased substantially, and unjustifiably. *Among many examples for groups of compounds which are included with no CAS numbers, as a single listing [1],* are the following: (1) Aminoglycosides; (2) Anabolic steroids; (3) Aristolochic acids; (4) Barbiturates; (5) Benzidine-based dyes; (6) Benzodiazepines; (7) Cadmium compounds; (8) Chromium (hexavalent compounds); (9) Conjugated estrogens; (10) 3,3'-Dimethoxybenzidine-based dyes; (11) 3,3'-Dimethylbenzidine-based dyes; (12) Dinitrotoluene; (13) Estrogens; (14) Hexachlorocyclohexane; (15) Lead compounds; (16) Nickel compounds; (17) Polychlorinated dibenzo-p-dioxins; (18) Polychlorinated dibenzofurans; and (19) Tetracyclines. The pertinent explanation is provided in the Proposition 65 listing ([1]: page 1, lines 10/11) that reads as follows:

No CAS number is given when several substances are presented as a single listing.

I also disagree with Dr. Chris Mackay's statement that " … *Alternately, it could refer to a specific congener of diaminotoluene (e.g. 2,4-diaminotoluene) but refer to a mixture of 2,4-diaminotoluene salts (chlorides, sulfates, phosphates, carbonates, etc) since all diaminotoluene congeners are weak bases*" ([2], Exhibit A:, p.3, lines 13-16). The document that forms the basis for DAT listing ([3], *Exhibit* B) does not include any statement about salts of DAT isomers. In fact, it is an omission that needs to be corrected since salts of all DAT isomers should be able to release free bases under physiological conditions. At the end of this document, suggestions are made as to how to further clarify the DAT listing, in particular, by introducing the DAT salts as well.

11. ***Declaration, entry 10.*** *CAS numbers are assigned to uniquely describe single chemical compounds or common mixtures with chemical, commercial, or regulatory importance. Review of the EPA document that forms the bases of the Proposition 65 listing shows the number 77 entry for diaminotoluene (mixed) identifies the material specifically as CAS number 00095807 (Exhibit 2). This number identifies specifically the free base of the 2,4-diaminotoluene congener. Consequently, to be consistent with the EPA document, it is my opinion that the most correct definition of diaminotoluene (mixed) is a formulation of mixed salts of 2,4-diaminotoluene. At the very least, the mixture must contain in part a component of 2,4-diaminotoluene. Otherwise the mixture has no relation to the chemical identified by EPA as 0009587; 2,4-diaminotoluene.*

Rebuttal. In entry 10 of this declaration I have already addressed this issue in great detail. In addition, the statement above "… most correct definition of diaminotoluene (mixed) is a formulation of mixed salts of 2,4-diaminotoluene … " is unfounded because in the basis document the DAT *salts* are not discussed at all [3] (*Exhibit B*), and it is explained that the "Diaminotoluene (mixed)" listing " … based on the carcinogenic properties of the isomer 2,4-diaminotoluene … **applicable to all isomers of diaminotoluene**."

Also incorrect is the statement by Dr. Chris Mackay (above) that "At the very least, the mixture must contain in part a component of 2,4-diaminotoluene." The document that forms the basis for the "Diaminotoluene (mixed)" listing does not indicate – implicitly, or explicitly - that 2,4-DAT must be present in order to qualify any formulation for the Proposition 65 warning. The summary statement "… **applicable to all isomers of diaminotoluene** …" means wherever *any of the diaminotoluene isomers are present – individually, or in combination with any other isomers, in any proportions – the Proposition 65 warning must be given.*

Part III. Scientific Evidence on the Toxicity and Carcinogenic Hazards of Diaminotoluenes

12. I have reviewed the OEHHA's recent call for information on the toxicity of isomeric

diaminotoluenes [4], and, given my scientific background, I consider my civic duty to opine. Below is the summary on toxicity of isomeric DATs, along with suggestions as to how to clarify the Proposition 65 scope of listing in order to make it explicit, unambiguous, and scientifically sound. The said recommendations will remove grounds for misinterpretations and facilitate enforcement of Proposition 65 in future litigation cases.

13. Toxicity of 2,5-Diaminotoluene (2,5-DAT; CAS No. 95-70-5).

2,5-Diaminotoluene was tested for mutagenicity by using a bacterial test specifically designed for detecting chemical mutagens [5]. The experimental conditions mimicked the actual use of "permanent," or oxidative type hair dyes containing diamines, such as 2,5-DAT, i. e. dye component is first activated with a strong oxidant (H_2O_2), then *in situ* formed colored molecules are applied to hair surface. ***In the case of 2,5-DAT, upon oxidation, microsomes-assisted mutagenicity increased 40-fold, making it strongly mutagenic*** [5] (*Exhibit C*). In human tests, 2,5-DAT was also proven to easily penetrate skin when applied to human scalp; its amount was quantified by measuring the 2,5-DAT diacetyl derivative in urine of human volunteers [5] (*Exhibit C*). The test in question measures mutagenesis by examining the specific base sequences, for example, the C-G-C-G-C-G-C-G sequence in select bacterial strain (*Salmonella typhimurium,* TA1538), meaning that it does not necessarily determine overall mutagenic potential of tested compounds and the actual "damage" can in fact be much higher.

The fact that 2,5-DAT is an established mutagen means that the component of hair dye can alter the genetic material, such as DNA, introducing mutations above the natural background level. It is well known in contemporary oncology that an increased frequency of mutations can cause cancer. This is the reason why mutagens are likely carcinogens, and many carcinogens are also shown to be mutagens. For example, *2,4-DAT, a Proposition 65 listed carcinogen (CAS No. 95-80-7), exhibits mutagenicity <u>under the same conditions</u> that were used in 2,5-DAT testing [5]* (*Exhibit C*). To further emphasize the link between mutagenicity and carcinogenicity, it should be mentioned that X-ray irradiation that undoubtedly causes cancer in humans is also shown to be mutagenic [6]. Its impact on fruit

flies resulted in genetic mutations producing mutants along with noticeable changes to chromosomes [6]. Furthermore, UV radiation, another known carcinogen, was proven to have a mutational effect as well [7]. Identifying chemical mutagens has become most efficient since the development of the Ames test in the 1970s that establishes mutagenicity as the preliminary step in identifying chemical carcinogens [8,9]. ***Extensive screening of chemical compounds indicated that some 90% of known carcinogens are also mutagenic [10-12], and around 80% of known mutagens may also be carcinogens [12,13].*** How intertwined, although not identical, the "mutagenicity" and "carcinogenicity" are can be seen even from the tiles of these seminal publications, i. e. "…Detection … of Mutagens and Carcinogens …", "… Chemicals Causing Mutations and Cancer … ", and "… Carcinogenicity Assessment … Based on … Mutagenicity."

Since 2,5-DAT is established to be mutagenic [5] (*Exhibit C*), then, by definition, it is also ***genotoxic***. In fact, ***all mutagens are genotoxic, but not all genotoxic compounds are mutagenic***. The reason is that the type of damage for genotoxic materials, by its spectrum, and type of damage to cells, is different from typical mutagenicity. These properties have so much in common, that the Ames test for mutagenicity, known as *Bacterial Reverse Mutation Assay* [8], is also used as genotoxicity assay in order to detect point mutations, such as a frame shift and base substitution. Overall, genotoxic agents induce damage to the DNA sequence and structure, thus incurring DNA lesions, which in turn lead to carcinogenesis. A typical example of chemical compounds which are *genotoxic, mutagenic*, and *carcinogenic* are pyrrolizidine alkaloids isolated from plants [14]. When metabolically activated, they are genotoxic, producing DNA adducts, DNA breaks, DNA cross-linking, and chromosomal aberrations. As mutagens, they cause mutations within the gene sequence both *in vivo* and *in vitro,* and are responsible for liver carcinogenesis.

Further evidence on the hazardous nature of 2,5-diaminotoluene can be found in the extensive report prepared by the European Union's Scientific Committee on Consumer Safety titled "Opinion on Toluene-2,5-diamine and its Sulfate" [15] (*Exhibit D*). This report includes some data from an in-depth analysis carried out by NIH and summarized in an

earlier work titled "Bioassay of 2,5-Toluenediamine Sulfate for Possible Carcinogenicity" [16] (*Exhibit E*). Below are some selections from reference [15] pertaining to mutagenicity, genotoxicity, or carcinogenicity of 2,5-DAT.

Excerpt #1 ([15], *Exhibit D*, p.34): In Bacterial Reverse Mutation Test, … toluene-2,5-diamine sulfate was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from phenobarbital/β- naphthoflavone-induced rats was used as exogenous metabolic activation system. … Conclusion: Under the experimental conditions used ***toluene-2,5-diamine sulfate was genotoxic (mutagenic)*** in this gene mutation test in bacteria.

Excerpt #2 ([15], *Exhibit D*, p.36): In *in vitro* Chromosome Aberration Tests on Sprague-Dawley rats or Golden Syrian hamsters, … toluene-2,5-diamine sulfate was investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in V79 cells. … Conclusion: Under the experimental conditions used the increase in cells with structural chromosomal aberrations indicates a ***genotoxic (clastogenic) activity of toluene-2,5-diamine sulfate.***

Excerpt #3 ([15], *Exhibit D*, p.36/37): In *in vitro* unscheduled DNA synthesis test, … 2,5-diaminotoluene was investigated for the induction of unscheduled DNA synthesis (UDS) in primary hepatocytes isolated from rats and hamsters. … Conclusion: Under the experimental conditions used ***2,5-diaminotoluene induced unscheduled DNA synthesis*** and, consequently, is genotoxic in rats in this in vitro UDS test.

Excerpt #4 ([15], *Exhibit D*, p.40): In *in vivo* alkaline single cell gel electrophoresis (Comet) assay in mice and rats, … 2,5-diaminotoluene sulfate was investigated for the induction of DNA damage in the alkaline single cell gel electrophoresis (Comet) assay in various tissues of mice and rats. … Conclusion: Under the experimental conditions used ***2,5-diaminotoluene sulfate induced significant DNA damage in the stomach of rats*** and, consequently, 2,5-diaminotoluene sulfate is genotoxic in the Comet assay with rats.

Excerpt #5 ([15], *Exhibit D*, p.43/44) ***Carcinogenicity study***: Toluene-2,5-diamine

sulfate was administered in the diet to groups of ***50 mice*** per sex at either 0.06 or 0.2% for a period of 78 weeks followed by an additional 16 to 19 weeks of observation. … A statistically significant increase in ***lung tumors*** (***alveolar/bronchiolar adenomas or carcinomas***) was observed in high dose female mice (high dose 8/45 (17%) vs. 1/45 (2%) in high dose control, P=0.016).

Excerpt #6 ([15], *Exhibit D*, p.44/45) ***Carcinogenicity study***: Toluene-2,5-diamine sulfate was administered in the diet to groups of ***50 rats*** per sex at either 0.05-0.06 or 0.2% for a period of 78 weeks followed by an additional 28 to 31 weeks of observation. … A statistically significant increase in the incidence of interstitial cell ***tumors of the testis*** was observed in male rats (low dose 43/48 (90%) vs. 33/45 (73%) in low dose control, P=0.039; high dose 47/48 (98%) vs. 19/24 (79%) in high dose control, P=0.014), but this was not considered treatment-related since the spontaneous incidence of these tumors in male rats is very high and variable. It has to be emphasized that although in the NCI report [16] (*Exhibit E*) the conclusion was drawn that "… under the conditions of this bioassay, sufficient evidence was not provided to conclusively demonstrate the carcinogenicity of 2,5-toluenediamine sulfate in either Fischer 344 rats or B6C3F1 mice," it is apparent that studies on mice and rats provide experimental evidence on carcinogenicity of 2,5-diaminotoluene. ***Even if this evidence is not considered to be "sufficient" by some strict statistical criteria, these data, in tandem with reported mutagenicity, genotoxicity and other relevant properties, provide, in my opinion, enough scientific grounds for inclusion of 2,5-diaminotoluene in the Proposition 65 list of carcinogens.***

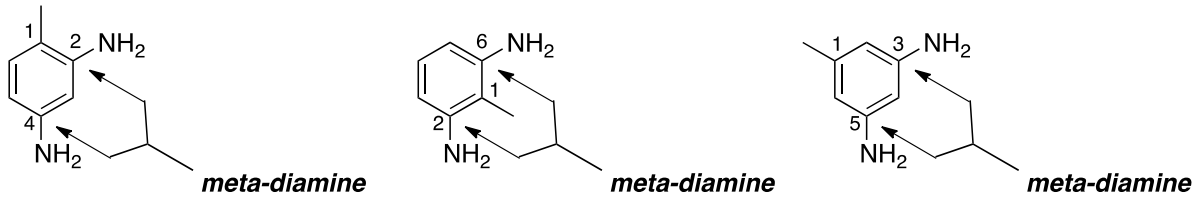
2,5-Diaminotoluene was also tested on mice for alleged teratogenicity [17] (*Exhibit F*). Animals were injected with hair-dye constituent, in a single dose, throughout the pregnancy, and fetuses were examined for possible abnormalities. A high incidence of skeletal malformations were discovered including " … fused or distorted thoracic vertebrae associated with absent or fused ribs … ." [17] (*Exhibit F*). Given the demonstrated mutagenicity of 2,5-DAT [5,8], it is suggested that the DNA of embryonic cells might be adversely impacted by the chemical, thus leading to observed teratogenicity. The authors

also raise the concern that the observed teratogenicity for the hair-dye constituent indicates that ***2,5-DAT can act as a reproductive toxicant and, when used by expectant mothers, the chemical could potentially cause birth defects.***

14. Toxicity of 2,6-Diaminotoluene (2,6-DAT; CAS No. 823-40-5) and 3,5-Diaminotoluene (3,5-DAT; CAS No. 108-71-4), and Their Relevance to 2,4-Diaminotoluene, a Listed Carcinogen.

The structures of three isomeric DAT derivatives are shown in Figure 1 with the first one, 2,4-DAT, being a proven, Proposition 65 listed carcinogen, and with the other two – 2,6-DAT and 3,5-DAT – currently under consideration [4]. All three of them have the same disposition of amino (NH_2) groups, i. e. meta- to each other. In other words, all three belong to the same class of organic compounds called aromatic meta-diamines. Since the biological properties are preponderantly determined by the amino groups, and their disposition around the aromatic framework, it is scientifically justified to assume that many biological properties will also be close to each other. The reason for this is that while interacting inside the human body with biological molecules (DNA, nucleic acids, peptides, amino acids, enzymes), the amino group is the main functional group that undergoes metabolic changes and converts into new derivatives that in turn are capable of triggering a physiological response [18] (*Exhibit G*). In other words, if 2,4-DAT, a meta diamine, is a listed compound, then other meta-diamines – 2,6-DAT and 3,5-DAT – should also be listed because the position of methyl groups around the aromatic ring should not substantially change the biological properties of compounds in question. To provide additional evidence that the presence of alkyl groups, such as a methyl group, around the aromatic nuclei does not qualitatively change their biological properties, in Figure 2, four groups of aromatic compounds are given, differing in the disposition of methyl groups around the rings. In Proposition 65 [1], benzidine skeleton is represented by the benzidine, a parent molecule, and its 3,3'-dimethyl derivative. Both are proven carcinogens listed along with their salts, and also dyes that contain a benzidine moiety. Also shown in Figure 2, are four anilines, all Proposition 65 listed compounds, which differ from each other by the number of methyl groups, and their disposition. It is additional proof that the biological response is mainly

determined by the aromatic amine moiety itself, and not by methyl substituents that still can have some incremental impact. The remaining two groups (benzene/toluene and benz[a]anthracene and 7,12-dimethylbenz[a]anthracene) are not DAT analogs as anilines are, but they still prove the point that introducing a methyl group(s) into a carcinogenic parent molecule does not eliminate its carcinogenicity. Benz[a]anthracene is a representative of Polynuclear Aromatic Hydrocarbon (PAH), a class of strongly



2,4-DAT

Prop 65 status:
listed

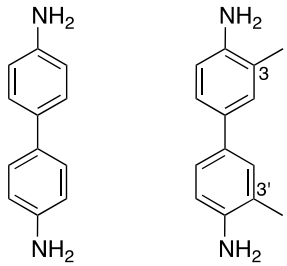
2,6-DAT

Prop 65 status:
under consideration

3,5-DAT

Prop 65 status:
under consideration

Figure 1.

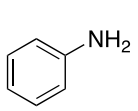


benzidine

Prop 65 status:
listed

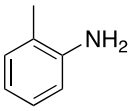
3,3'-dimethylbenzidine

Prop 65 status:
listed



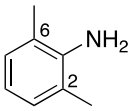
aniline

Prop 65 status:
listed



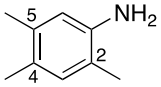
o-toluidine

Prop 65 status:
listed



2,6-xylidine, or
2,6-dimethylaniline

Prop 65 status:
listed

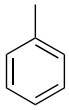


2,4,5-trimethylaniline

Prop 65 status:
listed

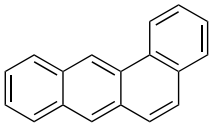
benzene

Prop 65 status:
listed



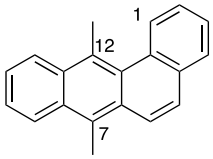
toluene

Prop 65 status:
listed



benz[a]anthracene

Prop 65 status:
listed



7,12-dimethylbenz[a]anthracene

Prop 65 status:
listed

Figure 2.

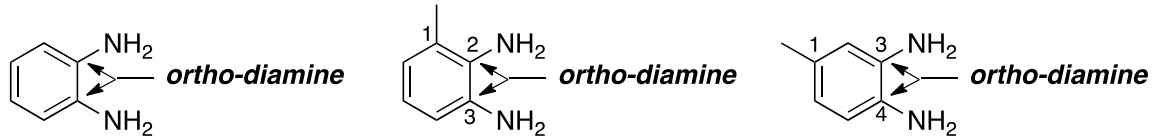
carcinogenic compounds [19], and its dimethyl derivative with two methyl groups in positions 7 and 12, is so powerful as a carcinogen that it serves in cancer research as a reference tumorigenic compound (abbreviated as DMBA).

2,6-Diaminotoluene exhibits mutagenicity in the Ames test on par with 2,4-DAT, a listed chemical [20] (*Exhibit H*). The difference in carcinogenic potential between 2,4-DAT and 2,6-DAT is explained by the relative ability to induce the CYP1A enzyme responsible for bioactivation of aromatic compounds. While 2,4-DAT is capable of autoinducing its own activation, it would be important to test the carcinogenic potential of 2,6-DAT *in the presence of the CYP1A enzyme*. In other words, testing their carcinogenicity should not be dependent upon their relative abilities to increase the expression of the metabolizing CYP1A enzyme. Genotoxic studies on 2,6-DAT revealed that although inferior to that of 2,4-DAT, it is capable of inducing the formation of DNA adducts that in turn constitutes a triggering step in the formation of tumors [21] (*Exhibit I*). It is worthy to mention that the DNA adducts with 2,6-DAT were detected *even after a single dose treatment* indicating that with the chronic, more extended exposure to 2,6-DAT the amount of DNA adducts, and the chances to develop cancer, will rise. The relationship between *in vitro* mutagenicity and *in vivo* carcinogenicity is a complex scientific issue [22] (*Exhibit J*) that requires additional studies on 2,6-DAT like chemicals; meanwhile, there are enough scientific grounds to include 2,6-DAT in the Proposition 65 list as a potential carcinogen. Another direct indication of an intrinsic hazardous nature of 2,6-DAT is its proven ability to produce free radical species in yeast, that when quantified by means of fluorescence spectroscopy, appeared to be much higher than the radical damage induced by carcinogenic 2,4-DAT [23].

15. Toxicity of 2,3-Diaminotoluene (2,3-DAT; CAS No. 2687-25-4) and 3,4-Diaminotoluene (3,4-DAT; CAS No. 496-72-0).

o-Phenylenediamine and its salts are included in Proposition 65 as carcinogens since May 15, 1998 (Figure 3). The presence of one amino group attached to the aromatic ring forms carcinogenic aromatic amines, or arylamines, which already have 19 (!) representatives in the current Proposition 65 list [1] (please see entries 16 and 17).

Introducing the second amino group, ortho to the first one, creates a qualitatively new entity that can undergo a different type of metabolism inside the human body and exhibit carcinogenicity. *o*-Phenylenediamine, a listed chemical, belongs to the class of ortho-diamines (Figure 3) which can metabolically be oxidized to ortho-benzoquinone diimine, which in turn can form DNA conjugates thus irreversibly damaging its structural integrity. There is a very significant knowledge base on this class of compounds, in particular, for their oxygen analogs which are metabolically formed from female hormones. Several derivatives are already listed chemicals - estradiol 17□, estrogens, estrone, ethinylestradiol [1] - shown to react with DNA bases and form DNA conjugates with some 40 of them being isolated and structurally characterized. 2,3-DAT and 3,4-DAT are direct structural analogs of o-phenylenediamine because of ortho disposition of both amino groups (Figure 3). The presence of a methyl group around the aromatic nucleus in 2,3-DAT and 3,4-DAT is unlikely to qualitatively change the biological properties and metabolic patterns of the parent compound, ortho-phenylenediamine. In Figure 2, multiple examples are given to demonstrate that if the parent compound is carcinogenic, then, even with the methyl group(s) introduced to the aromatic nucleus, its alkylated derivatives still retain carcinogenicity. Subsequently, ***since ortho-phenylenediamine is a carcinogen included in the Proposition 65 list, then its mono-methylated analogs - 2,3-DAT and 3,4-DAT – with the <u>same disposition of amino groups</u> - ortho toward each other - should also be included in the Proposition 65 list.***



o-phenylenediamine	2,3-DAT	3,4-DAT
Prop 65 status: **listed**	Prop 65 status: **under consideration**	Prop 65 status: **under consideration**

Figure 3.

16. Carcinogenicity of Aromatic Amines as an Intrinsic Property. Aromatic amines are proven urinary bladder carcinogens in men and are also reported to induce tumors at multiple locations in laboratory animals including liver, intestines, and mammary gland [18] (*Exhibit G*). Metabolic activation of aromatic amines is well understood and involves multiple enzymatic steps with each of them capable of altering the structural integrity of DNA by forming respective DNA conjugates. Among them are the following: (a) *ortho*-hydroxylation of the aromatic ring, subsequent conversion to *ortho*-benzoquinone imine, and formation of DNA conjugates at *meta*-position of the aromatic nucleus (*nucleophilic addition mechanism*); (b) hydroxylation of the amino group and formation of DNA conjugates either at N-terminus, or an *ortho*-position of the aromatic ring (*nucleophilic substitution mechanism*); and (c) N-acetylation followed by the formation of DNA conjugates at the N-terminus (*nucleophilic substitution mechanism*). Among compounds tested are those present in the Proposition 65 listing such as 1-naphthylamine, 2-naphthylamine, 4-aminobiphenyl, 2-acetylaminofluorene, and benzidine [18] (*Exhibit G*).

A long-term impact of aromatic amines on human health is further corroborated by epidemiological studies reporting on ***increased incidence of bladder cancer in dyestuff workers even after 30 years of exposure*** [24]. Most alarmingly, the risk of bladder cancer is higher when the exposure to aromatic amines occurred at a younger age. More detailed analysis on the carcinogenicity of aromatic amines, and underlying intimate mechanistic details, can be found in IARC monograph [25] (*Exhibit K*).

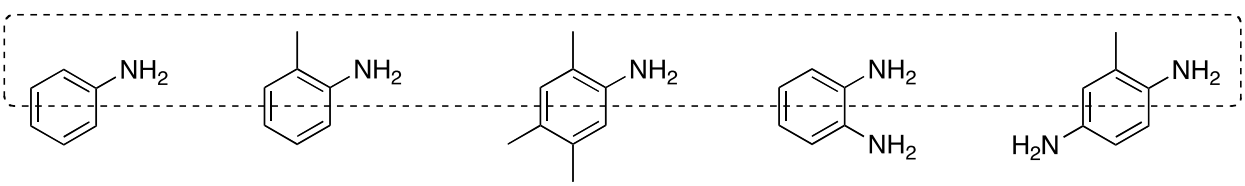
17. Representation of Aromatic Amines in Proposition 65 Listing. Given the very nature of aromatic amines capable of metabolizing by multiple routes by body enzymes, forming DNA adducts, and exhibiting mutagenicity, genotoxicity, and carcinogenicity in humans as well as animals [18] (*Exhibit G*), it does not come as a surprise that the said class of organic compounds is heavily represented in the Proposition 65 listing of proven carcinogens [1]. Below is the compilation of aromatic amines which are different by their molecular weight, molecular structure, complexity, functionality, topology, and positional isomerism, but they all have a common structural feature, i. e. *at least one amino group*

directly attached to the benzene ring.

(1) Aniline;
(2) 2-Aminofluorene;
(3) 1-Amino-2-methylanthraquinone;
(4) 4-Amino-2-nitrophenol
(5) o-Anisidine;
(6) Benzidine;
(7) Benzidine-based dyes;
(8) 3,3'-Dimethoxybenzidine;
(9) 3,3'-Dimethylbenzidine;
(10) Butylated hydroxyanisole;
(11) p-Chloroaniline;
(12) Diazoaminobenzene;
(13) 4,4'-Methylenedianiline;
(14) 1-Naphthylamine;
(15) 2-Naphthylamine;
(16) 2,4,5-Trimethylaniline.
(17) o-Toluidine;
(18) p-Chloro-o-toluidine;
(19) 5-Chloro-o-toluidine.

All these carcinogenic aromatic amines are structurally related to isomeric diaminotoluenes (Figure 4). Aniline, a parent molecule of aromatic amines, is present, as a structural unit, in all DAT isomers. Methyl groups introduced in different positions of the aromatic nucleus do not change the intrinsic nature of the compound, i. e. analogous to aniline, o-toluidine and 2,4,5-trimethylaniline both are listed as carcinogens [1]. Another pair of listed compounds which are different in a degree of substitution with methyl groups are benzidine and 3,3'-dimethylbenzidine derivative ([1]; Figure 2). The reason for this phenomenon is that the presence of relatively small methyl groups (26Å^3) does not qualitatively affect either major metabolic pathways of xenobiotics, or their structural,

conformational, and electronic characteristics, thus allowing them to retain carcinogenic properties of parent compounds. The incorporation of the second NH_2-group to the aromatic nucleus forms another listed carcinogen, o-phenylenediamine (Figure 4). Isomeric DATs, such as 2,5-diaminotoluene, represent a structural combination of aniline and toluene molecules with a methyl group(s) and one, or two, NH_2-groups positioned around the periphery of the aromatic framework. It does not come as a surprise that 2,5-DAT also exhibits mutagenicity, genotoxicity, and carcinogenicity analogous to other aromatic amines. The point here is that the ***aromatic amines are inherently harmful to the human body, and with at least 19 representatives already included in the Proposition 65 list of carcinogens***, the related structures, such as 2,5-DAT, or other isomers of diaminotoluene, should also be included if we are to properly protect the unsuspecting general public from harmful chemicals. In fact, even 26 years ago, in 1988, the Cal EPA OEHHA made the right decision to list *all* DAT isomers in Proposition 65 [3] (*Exhibit B*). It was the right decision then, and it is even more justified today to keep all DAT isomers listed given the experimental data, existing knowledge base, current understanding of metabolism, and carcinogenicity of close structural analogs.



aniline	o-toluidine	2,4,5-trimethylaniline	o-phenylenediamine	2,5-DAT
Prop 65 status: **listed**	Prop 65 status: **listed**	Prop 65 status: **listed**	Prop 65 status: **listed**	Prop 65 status: **under consideration**

Figure 4.

Part IV. Recommendations

18. The current description of diaminotoluene (mixed) in the Proposition 65 List of Chemicals is adequate and includes "all isomers of diaminotoluene" as accurately explained in the U. S. EPA basis document for listing titled "Evaluation of the Potential Carcinogenicity of Diaminotoluene (Mixed) (95-80-7)" [3] (*Exhibit B*). The CAS number is not given in

Proposition 65 because several substances are presented as a single listing. The clarification is given on the first page of the Proposition 65 listing ([1]; lines 10/11).

Some modifications can nevertheless be introduced to further clarify the scope of listing, and also reflect on the wealth of scientific evidence that came to light during the last decade. I recommend that the Proposition 65 listing pertaining to diaminotoluenes be modified as follows:

Current listing	***Suggested listing***	***Type of Toxicity***
Diaminotoluene (mixed)	Diaminotoluene isomers and their salts	cancer

Also, given the demonstrated teratogenicity of 2,5-diaminotoluene [17] (*Exhibit F*), the latter should be included into the Proposition 65 list as a reproductive toxicant.

Current listing	***Suggested listing***	***Type of Toxicity***
None	2,5-Diaminotoluene and its salts	developmental

The interpretation provided by the Carcinogen Identification Committee (CIC) of OEHHA's Science Advisory Board that serve as the State's Qualified Experts (SQE), and will soon be rendering an opinion with respect the DAT listing, should explicitly state the following:

> ***"Diaminotoluene isomers"*** in the Proposition 65 listing include 2,3-diaminotoluene (CAS No. 2687-25-4), 2,5-diaminotoluene (CAS No. 95-70-5), 2,6-diaminotoluene (CAS No. 823-40-5), 3,4-diaminotoluene (CAS No. 496-72-0), and 3,5-diaminotoluene (CAS No. 108-71-4), that can be found in the consumer products either individually, or as mixtures, of any combinations thereof. 2,4-diaminotoluene (CAS No. 95-80-7) is not included in this single listing because it has been listed separately.

The phrase **"...and their salts"** in this single listing refers to organic and inorganic salts of diaminotoluene structural isomers listed above. Specifying the CAS numbers for salts is not possible, and unnecessary, given the large number of organic and inorganic acids that can potentially form salts with diaminotoluene isomers. The common feature that these salts have is that they can release diaminotoluene isomers, as free bases, under physiological conditions.

Part V

19. I reserve the right to modify my opinions as new information is discovered or brought to my attention.

20. As an expert, I am available for taking part in public hearings, or expert panel discussions, as well as for presenting findings to the Carcinogen Identification Committee of OEHHA's Science Advisory Board.

I declare under penalty of perjury under the laws of the State of California that the foregoing is true and correct. This declaration was executed on this 22nd day of December, 2014 in Northridge, Los Angeles County, California.

Dated 12.22.14

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References

[1] Chemicals Known to the State to Cause Cancer or Reproductive Toxicity. State of California, Environmental Protection Agency, Office of Environmental Health Hazard Assessment, Safe Drinking Water and Toxic Enforcement Act of 1986, Proposition 65, June 6, 2014.

[2] Petition of Big Lots Stores, Inc. to Reconsider the Proposition 65 Listing of Diaminotoluene (mixed) as a Carcinogen Pursuant to Proposition 65's Authoritative Body Mechanism; Sedgwick, LLP, San Francisco, CA, October 21, 2014; *Exhibit A*: Declaration of Dr. Chris Mackay.

[3] "Evaluation of the Potential Carcinogenicity of Diaminotoluene (Mixed) (95-80-7)" Carcinogen Assessment Group, Office of Health and Environmental Assessment under U. S. Environmental Protection Agency (EPA; 600/8-91/103); June 1988.

[4] "Request for Relevant Information on the Carcinogenic Hazards of Diaminotoluenes" published by Office of Environmental Health Hazard Assessment (OEHHA) on October 23, 2014.

[5] Ames, B. N.; Kammen, H. O.; Yamasaki, E. Hair Dyes Are Mutagenic: Identification of a Variety of Mutagenic Ingredients. *Proc. Nat. Acad. Sci. USA* **1975**, 72, 2423-2427.

[6] Muller, H. J. Artificial Transmutation of the Gene. *Science* **1927**, *66*, 84-87.

[7] Altenberg, E. The Limit of Radiation Frequency Effective in Producing Mutations. *The American Naturalist* **1928**, *62*, 540-545.

[8] Ames, B. N.; Lee, F. D.; Durston, W. E. An Improved Bacterial Test System for the Detection and Classification of Mutagens and Carcinogens. *PNAS* **1973**, *70,* 782-786.

[9] Ames, B. N. Identifying Environmental Chemicals Causing Mutations and Cancer. *Science* **1979**, *204*, 587-593.

[10] McCann, J.; Choi, E.; Yamasaki, E.; Ames, B. N. Detection of Carcinogens as Mutagens in the Salmonella/microsome Test: Assay of 300 Chemicals. *PNAS* **1975**, *72,* 5135-5139.

[11] McCann, J.; Gold, L. S.; Horn, L.; McGill, R.; Graedel, T. E.; Kaldor, J. Statistical Analysis of Salmonella Test Data and Comparison to Results of Animal Cancer Tests. *Mutation Research* **1988**, *205*, 183-195.

[12] Dunkel, V. C.; Zeiger, E.; Brusick, D.; McCoy, E.; McGregor, D.; Mortelmans, K.; Rosenkranz, H. S.; Simmon, V. F. Reproducibility of Microbial Mutagenicity Assays: II. Testing of Carcinogens and Noncarcinogens in Salmonella typhimurium and Escherichia coli. *Environ. Mutagenesis* **1985**, *7,* 1-248.

[13] Benigni, R.; Bossa, C. Alternative Strategies for Carcinogenicity Assessment: an Efficient and Simplified Approach Based on in vitro Mutagenicity and Cell Transformation Assays. *Mutagenesis* **2011**, *26,* 455-460.

[14] (a) Smith, L. W.; Culvenor, C. C. J. Plant sources of hepatotoxic pyrrolizidine alkaloids. *J. Nat. Prod.* **1981**, *44*, 129-15; (b) Schoental, R.; Kelly, J. S. Liver Lesions in Young Rats Suckled by Mothers Treated with the Pyrrolizidine (Senecio) Alkaloids, Lasiocarpine and Retrorsine. *The Journal of Pathology and Bacteriology* **1959**, *2*, 485–495. (c) Chen, T. Genotoxicity of Pyrrolizidine. *Applied Toxicology* **2010**, doi 10.1002/jat.1504. (d) Mei, N. Metabolism, Genotoxicity, and Carcinogenicity of *Comfrey*. *J. Toxicol. Environ. Health* **2010**, doi 10.1080/10937404.2010.509013. (e) Schoental, R. Toxicology and Carcinogenic Action of Pyrrolizidine Alkaloids. *Cancer Research* **1968**, 2237-2246. (f)

Naturally Occurring Pyrrolizidine Alkaloids. CRC Press, Boca Raton, FL, USA; 1990.

[15] Opinion on Toluene-2,5-diamine and its Sulfate, Scientific Committee on Consumer Safety (SCCS), European Commission, 72p, June 2012.

[16] Bioassay of 2,5-Toluenediamine Sulfate for Possible Carcinogenicity, National Cancer Institute, Carcinogenesis Technical Report Series, No. 126, (NCI-CG-TR-126), U. S. Department of Health, Education, and Welfare, Public Health Service, NIH; 112p, 1978.

[17] Inouye, M.; Murakami, U. Teratogenicity of 2,5-Diaminotoluene, a Hair-Dye Constituent, in Mice. *Fd. Cosmet. Toxicol.* **1977**, *15,* 447-451.

[18] Beland, F. A.; Kadlubar, F. F. Formation and Persistence of Arylamine DNA Adducts in Vivo. *Environmental Health Perspectives* **1985***, 62,* 19-30*.*

[19] (a) Heidelberger, C. Chemical Carcinogenesis. *Annu. Rev. Biochem.* **1975**, *44*, 79-121 and references cited therein. (b) Sims, P.; Grover, P.; Swaisland, A.; Pal, K.; Hewer, A. Metabolic Activation of Benzo[a]pyrene Proceeds by a Diol-Epoxide. *Nature (London)* **1974**, *252*, 326-328.

[20] Cheung, Y. L.; Shelling, J.; Mohammed, N. N. D.; Gray, T. J. B.; Ioannides, C. Interaction with the Aromatic Hydrocarbon Receptor, CYP1A Induction, and Mutagenicity of a Series of Diaminotoluenes: Implications for their Carcinogenicity. *Toxicol. Appl. Pharmacol.* **1996**, *139,* 203-211.

[21] Taningher, M.; Peluso, M.; Parodi, S.; Ledda-Columbano, G. M.; Columbano, A. Genotoxic and Non-genotoxic Activities of 2,4- and 2,6-Diaminotoluene, as Evaluated in Fischer-344 Rat Liver. *Toxicology* **1995**, *99*, 1-10.

[22] Zeiger, E. Mutagens That Are Not Carcinogens: Faulty Theory or Faulty Tests.

Mutation Research **2001**, *492*, 29-38.

[23] Brennan, R. J.; Schiestl, R. H. Diaminotoluenes Induce Intrachromosomal Recombination and Free Radicals in *Saccharomyces cerevisiae*. *Mutation Research* **1997**, *381*, 251-258.

[24] Pira, E.; Piolatto, G.; Negri, E. Bladder Cancer Mortality of Workers Exposed to Aromatic Amines: a 58-Year Follow-up. *Journal of National Cancer Institute* **2010**, *102*, 1-4.

[25] General Discussion of Common Mechanisms for Aromatic Amines in: *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans; Some Aromatic Amines, Organic Dyes, and Related Exposures*, Vol. 99, **2010**, 41-54.

EXHIBIT A

CURRICULUM VITAE

MELIKYAN, Gagik G., Professor of Chemistry, Department of Chemistry and Biochemistry, California State University Northridge (CSUN), Northridge, CA; tel. off. 818-677-2565; email: gagik.melikyan@csun.edu; research website: www.csun.edu/science-mathematics/chemistry-biochemistry/gagik-melikyan; book website: www.csun.edu/gmelikyan.

RESEARCH INTERESTS

Radical reactions of transition metal-complexed unsaturated systems; synthetic methodologies in organic and organometallic chemistry; novel therapeutic agents for a breast cancer treatment; enzymatic chemistry; cancer drug development; aromatase metabolism; hormonal chemistry; food chemistry; antioxidants; supplements; cosmetics; environmental chemistry, consumer product chemistry, environmental laws.

CONSULTING AND EXPERT SERVICES

2006-to-date provided litigation support to consumer protection groups; served as an expert witness in toxic consumer product torts (cosmetics; foods; electronics; petrochemicals); supported non-profit organizations in the "failure to warn" litigations and the state actions initiated under the California Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65); petitioned government agencies on listed carcinogens and reproductive toxins; provided consulting services to cosmetic industry on cosmetic formulations; rendered consulting services on environmental pollution related to irresponsible mining practices and environmental product liability cases; provided technical guidance on sampling protocols for environmental pollutants; prepared expert declarations; provided trial depositions; developed wiping protocol for phthalates; conducted human exposure and health risk analysis; evaluated interactions between the commercial products and consumer population; quantified the exposures to listed chemicals from materials in dispute according to NSRL and MADL standards.

PUBLICATIONS & BOOKS & PATENTS & PRESENTATIONS

82 papers in peer-reviewed journals, including 8 reviews/book chapters;
1 book;
3 patents;
120 presentations, including:
- national and international conferences – 61 (7 - invited);
- invited talks at academic institutions – 16;
- invited public presentations – 29;
- radio interview – 9;
- TV interviews – 5;

5 chairing sessions at ACS national and regional conferences.

SCIENTIFIC CITATION SCORES (as of 05.09.14)

874	sum of the times cited;
626	sum of times cited without self-citations;
17	h-index.

PUBLIC OUTREACH: EXPLAINING CHEMISTRY TO THE GENERAL PUBLIC

19,188	number of views of the health-related youtube videos.

PROFESSIONAL EXPERIENCE

1978-1990	Institute of Organic Chemistry, National Academy of Sciences, Yerevan, Armenia: Principal Researcher (1990); Senior Research Fellow (1983-1990); Junior Research Fellow (1978-1983); Research Group Leader (1978-1990);
1990-1992	Alexander von Humboldt Fellow, Institute of Organic Chemistry, University Erlangen-Nurnberg, Erlangen, Germany;
1993-1995	Adjunct Professor of Chemistry, Department of Chemistry&Biochemistry, University of Oklahoma, OK;
1995-1998	Assistant Professor of Chemistry, Department of Chemistry, CSUN, Northridge, CA;
1998-2003	Associate Professor of Chemistry, Department of Chemistry, CSUN, Northridge, CA;
2003-to-date	Professor of Chemistry, Department of Chemistry&Biochemistry, CSUN, Northridge, CA.

NEW REACTIONS DISCOVERED

1984	Pd-induced, one-step transformation of 5-alkyn-4-olids to 4E-alkenoic acids;
1990	oxidative methylene-extrusion reaction converting 2H-pyrans into furan derivatives;
1997	conversion of propargyl alcohols into cyclic and acyclic 1,5-alkadiynes mediated by heteroatom-containing organic molecules;
2003	spontaneous generation and coupling of Co-complexed propargyl systems.

INVITED PRESENTATIONS AT CONFERENCES (from a total of 7)

1999	35th ACS Western Regional Meeting, 1999 Pacific Conference, Ontario CA;
2000	American-German-French Symposium "Carbon-Rich Organometallics", Erlangen, Germany;
2002	National Foundation on Science & Advanced Technologies (NFSAT)-Civilian Research & Development Foundation (CRDF, Washington) 5th Anniversary Conference, Yerevan, Armenia;
2008	International Organometallics Conference 2008, ZING Conferences, Cancun, Mexico;
2009	General Assembly, National Academy of Sciences, Yerevan, Armenia.

CHAIRING SESSIONS AT ACS CONFERENCES

1998	"Organometallic Synthesis," 34th ACS Western Regional Meeting, 1998 Pacific Conference, San-Francisco, CA.

2011	"New Reactions and Methodology," 241st ACS National Meeting, Anaheim, CA.
2012	"Metal-Mediated Reactions and Syntheses," 243st ACS National Meeting, San Diego, CA.
2013	"Metal-Mediated Reactions and Syntheses," 245st ACS National Meeting, New Orleans, GA.
2014	"Asymmetric Reactions and Syntheses," 246st ACS National Meeting, San Francisco, CA.

INVITED PRESENTATIONS AT ACADEMIC INSTITUTIONS (from a total of 16)

UC San Diego, CA (1997); CSU Los Angeles, CA (1998); USC, Los Angeles, CA (1999); UC Riverside, CA (1996); University of Oklahoma, Norman, OK (1993); University of Erlangen-Nurnberg, Germany (1992); University of Hamburg, Germany (1991); University of Hannover, Germany (1991); Institute of Organic Chemistry, NAS, Yerevan, Armenia (1991); Department of Natural Sciences, NAS, Yerevan, Armenia (1998, 2009); University of Pierre and Marie Curie, Paris, France (2001); CSU Long Beach, CA (2009); California State University Northridge, Northridge, CA (2014); University of Oklahoma, Norman, OK (2014).

DISTINCTIONS & RECOGNITIONS

2002	*Polished Apple Award*, University Ambassadors, CSUN;
2008	2008 Outstanding Faculty Award, CSUN;
2008	*Foreign Member*, National Academy of Sciences, Yerevan, Armenia;
2010	Jerome Richfield Research Scholar Award, CSUN.

LITERARY AWARDS

2011	*Finalist* in the "Science" category, The USA "Best Books 2011" Awards competition sponsored by the USA Book News;
2012	*Second Place Award* in the "Science" category, 2012 International Book Awards competition sponsored by JPX Media.

AWARDS & HONORS

1970-1973	"Best Student of the Department of Chemistry", Department of Chemistry, Yerevan State University, Yerevan, Armenia;
1987	Deutscher Akademischer Austauschdienst (DAAD), Germany;
1990	Alexander von Humboldt Fellowship, Germany;
1997	Collaborative Research Award, Civilian Research and Development Foundation (CRDF);
1998	Hewlett Packard Corporation;
1998	Judge Julian Beck Foundation;
2001	NATO, Physical and Engineering Science and Technology;
2002	American Chemical Society, Petroleum Research Fund;
2006	America's Registry of Outstanding Professionals 2005-2006;
2006	Who's Who in Sciences Higher Education (WWSHE);
2007,2011	National Science Foundation (NSF), Research at Undergraduate Institutions (RUI).

EDUCATION

1973	B.S. in Chemistry, Yerevan State University, Yerevan;
1977	Ph.D. in Organic Chemistry, Institute of Elementorganic Compounds, National Academy of Sciences, Moscow;
1990	D.Sc. (Doctor of Scienecs) in Organic/Organometallic Chemistry, Institute of Organic Chemistry, National Academy of Sciences, Yerevan;
1990-1992	Alexander von Humboldt Fellow in Organic/Organometallic Chemistry, Institute of Organic Chemistry, University Erlangen-Nurnberg, Erlangen.

TEACHING RESPONSIBILITIES

Courses taught at CSUN since 1995 (12WTU/semester):

CHEM103	General Chemistry I;
CHEM235	Introductory Organic Chemistry (for non-Science majors);
CHEM331	Organic Chemistry I (for Chemistry majors);
CHEM332	Organic Chemistry II (for Chemistry majors);
CHEM331L	Organic Chemistry I Laboratory course;
CHEM332L	Organic Chemistry II Laboratory course;
CHEM333	Principles of Organic Chemistry I (for Science majors);
CHEM334	Principles of Organic Chemistry II (for Science majors);
CHEM333L	Principles of Organic Chemistry I Laboratory course;
CHEM334L	Principles of Organic Chemistry II Laboratory course;
CHEM595F	Organometalic Chemistry (for graduate students).

INDIVIDUAL STUDENT TRAINING & MENTORING

1984-1992	4 graduate students awarded Ph.D. degrees;
1999-2014	12 graduate students awarded *Master of Science* degrees;
1996-2014	graduate (up to 3/semester) and undergraduate (up to 6/semester) students enrolled in CHEM696, CHEM495, and CHEM499 courses (total number of students – 78, including 65 undergraduate students; total of 417 student-semesters including 258 undergraduate student-semesters);
1997-2014	104 student presentations (including 54 - by undergraduate students) at the departmental, school, university, state and national levels, including the ACS National and Regional Meetings, Southern California Conferences on Undergraduate Research, CSUN Student Research Symposia, CSU-Wide Students' Research Symposia, and Sigma Xi Competitions;
1996-2014	45 student awards (including 28 - by undergraduate students), such as Donald Bianchi Research Awards, Best Presentation, 1st, 2nd and 3rd Place Awards at Sigma Xi Competitions (CSUN, 1996, 1997, 1998, 1999, 2003, 2005, 2006, 2007, 2009, 2013); First Place Awards at CSUN Student Research Symposia (CSUN, 1996, 1997, 2004, 2005, 2007, 2010, 2013); Second Place Award at CSUN Student Research Symposium (CSUN, 2002; 2006, 2010, 2013, 2014); Second Place Award at CSU Student Research Competition (CSU Chico, 1998); Second Place Award at CSU Student Research Competition (CSU Hayward, 2008), Nathan O. Freedman Memorial Award (CSUN, 2010); Outstanding Junior Award (CSUN, 1998); Hypercube Scholar Award (CSUN, 1999); Organic Chemistry Award (CSUN, 2000, 2012); Outstanding Graduate Student Teaching Award (CSUN, 2005, 2007, 2009); Graduate Fellow Award for

Outstanding Research Promise in Science and Mathematics (CSUN, 2009); First Place Award, Award of Excellence, AAAS Pacific (San Francisco, 2010), Outstanding Freshman Chemistry Award (CSUN, 2011); Analytical Chemistry Award (CSUN, 2011); Henry Klostergard Award for Outstanding Graduating Chemistry Major (CSUN, 2012); Outstanding Graduating Senior Award (2014); 2014 Wolfson Award (2014).

1996-2013 27 student proposals (submitted by undergraduate students - 9; funded - 21), University Corporation, CSUN.

CURRICULUM DEVELOPMENT / PROFESSIONAL SERVICES

I. Revised the curriculum of Organic Chemistry laboratory for chemistry majors (CHEM331L/332L) bringing up the level of instruction to that in research laboratory;
II. Introduced high-resolution chromatographic methods and state-of-the-art experiments into Organic Chemistry curriculum;
III. Initiated and ran new seminar series for undergraduate and graduate students "Students' Research Seminar";
IV. Evaluator, Single-subject credential evaluation of science teacher;
V. Acted as a member, and chaired, the Curriculum Committee, College of Science and Mathematics, CSUN;
VI. Coordinated Organic Chemistry laboratory courses (CHEM235/333/334);
VII. Acted as a library liaison for Department of Chemistry&Biochemistry;
VIII. Continuously upgraded undergraduate Organic Chemistry lecture courses;
IX. Faculty Hearing Panel, CSUN;
X. Post Promotion Increase (PPI) Committee, Department of Chemistry, CSUN;
XI. Judge, 12th Annual Sigma Xi Student Research Symposium, CSUN;
XII. Member, Part-time faculty personnel committee, Department of Chemistry, CSUN;
XIII. Member, Department Chair Search and Screen Committee, Department of Chemistry, CSUN;
XIV. Member, Post-tenure faculty evaluation committee, Department of Chemistry, CSUN.

EXTRAMURAL FUNDING

2011 National Science Foundation (NSF) - $240K;
2007 National Science Foundation (NSF) - $240K;
2003 American Chemical Society, Petroleum Research Fund (ACS PRF) - $50K;
2001 NATO, Physical and Engineering Science and Technology - $7K;
1998 Hewlett Packard - $83K;
1997 Civilian Research & Development Foundation (CRDF) - $40K.

MEDIA COVERAGE

1994 The Norman Transcript, Norman, OK (article titled "Accelerated Course in Organic Chemistry Offered by University of Oklahoma);

1995-2005	SCOPE, College of Science&Math Newsletter (quarterly; multiple publication and presentation reports);
2003	Chemical and Engineering News, Science & Technology Concentrate page (Sept 22; p.28) featuring a new reaction discovered at CSUN and published in Organic Letters 2003, 5, 3395;
2004	CSUN Press Release "Cal State Northridge Chemistry Professor on a Quest to Defeat Breast Cancer";
2004	Northridge Magazine (article titled "Cancer Research and Outreach");
2005	New Times (Novoye Vremya) newspaper, Yerevan, Armenia; an interview on the US and Armenian educational systems and reorganization plan of the Armenian National Academy of Sciences;
2005	Northridge Magazine (article titled "Chemistry Professor on a Quest to Defeat Breast Cancer");
1998-2008	@CSUN newspaper (multiple presentation and publication reports);
2006-2008	CSUN web banner "Gagik Melikyan is on a mission to find a breast cancer cure";
2008	The Armenian Reporter, April 2008, No. 58, p.3;
2009	Asbarez daily newspaper, February 2, 2009; "ARPA Board Members Elected as Foreign Members of National Academy of Sciences of Armenia";
2009	@CSUN newspaper, January 20, 2009; "Chemistry Professor Elected to Armenian Science Academy";
2009	California Courier, February 5, 2009;
2009	The Armenian Reporter, February 14, 2009;
2009	The Armenian Observer, February 18, 2009;
2011	North Valley Community News, January 2011;
2011	The Armenian Observer, January 5, 2011;
2011	The California Courier, January 13, 2011;
2011	In Focus, California Writers Club / West Valley, February, 2011;
2011	Daily Sundial, CSUN, Northridge, CA; February 2, 2011;
2011	Community@CSUN, CSUN, Northridge, CA; February 2011 (Vol X, #5);
2011	@CSUN, CSUN, Northridge, CA;
2011	Northridge Magazine, Word's Out, #61, Summer 2011, CSUN, Northridge, CA;
2012	*Valley Scribe*, California Writers Club /San Fernando Valley, February, 2012;
2012	*USA Armenian Life* Magazine, March 23, 2012;
2012	Radio-TV Interview Report (RTIR), March-June 2012;
2012	*Chemical and Engineering News*, June 25, 2012;
2012	*Speaker Showcase*, Bradley Communications, Broomall, PA;
2013	*Discussing the Facts vs. Hypes of Antioxidants, Supplements and Cosmetics, CSUN Today,* Feb 25, 2013;
2013	Northridge Magazine, Spring 2013, CSUN, Northridge, CA;
2013	*Writer's Website of the Month*, The Valley Scribe, California Writers Club / West Valley, February, 2013;
2014	*CSUN SHINE*, Spring 2014, CSUN, Northridge, CA;
2014	*TheRoundUp*, May 2014, Pierce College, CA.

TV APPEARANCES

2009 AMGA channel, Glendale, CA;
2011 USArmenia channel, Burbank, CA;
2011 CBS KCAL9, Studio City, CA;
2012 USArmenia channel, Burbank, CA;
2013 The Book Beat, LA TALK LIVE channel, Los Angeles, CA.

RADIO INTERVIEWS

2012 DresserAfterDark, BBS Radio;
2012 KYNT KSWR radio;
2012 KCWR radio;
2012 KERN Bakersfield Good Morning News radio;
2012 WOCM radio;
2012 KCSN-FM radio;
2012 KFNX radio;
2012 IssuesToday radio;
2012 Dr. Joe Radio Show.

PUBLIC APPEARANCES (from a total of 29)

2002 Invited speaker, U.S. Civilian Research & Development Foundation (CRDF) and Armenian Diaspora meeting, Glendale, CA;
2007 Speaker, Alumni & Friends of Armenian Studies program (AFASP) banquet, CSUN, Northridge, CA;
2008 Speaker, Graduation Ceremony, Armenian Studies Program, CSUN, Northridge, CA;
2008 Speaker, 25th Anniversary Meeting of the Armenian Studies Program, CSUN, Northridge, CA;
2009 Invited speaker, Ferrahian High School, Tarzana, CA;
2009 Invited speaker, High School Junior Honor's Day, CSUN, Northridge, CA;
2010 Invited speaker, ARPA Institute, book presentation, Thousands Oaks, CA;
2011 Invited speaker, Ararat-Eskijian Museum, book presentation, Mission Hills, CA;
2011 Invited speaker, Phi Delta Epsilon, book presentation, CSUN, Northridge, CA;
2011 Invited speaker, Organization of Istanbul Armenians (OIA), Glendale, CA;
2011 Invited speaker, California Writers' Club, San Fernando Valley Chapter, Canoga Park, CA;
2011 Invited speaker, Mission Community Hospital, Panorama City, CA;
2011 Invited speaker, Provost Colloquium, Jeromy Richfield Award presentation, CSUN, Northridge, CA;
2011 Keynote Health Speaker, Filipino American Chamber of Commerce of Orange County (FACCOC), Garden Grove, CA;
2011 Keynote Health Speaker, *Staying Healthy and Insights into "Natural" in Health Foods, Remedies, and Cancer Prevention*. Inland Empire Asian Business Association (IEABA), Riverside, CA;
2011 Invited speaker, *Health is Wealth*. USANA Inc. Regional Center, Signal Hills, CA;

2012 Book Bazaar, California Writers' Club, San Fernando Valley, Woodland Hills, CA;

2012 Invited speaker, Armenian Engineers and Scientists of America (AESA), Glendale, CA;

2013 Panelist, California Writers' Club, San Fernando Valley, Woodland Hills, CA;

2013 Invited speaker, Student Organization for a Holistic Approach to Health and Leadership (SOHHL), Phi Delta Epsilon Fraternity, Chicanos for Community Medicine (CCM) Student Organization, CSUN, Northridge, CA;

2013 Guest Speaker, *Women's History Month event*, United States Citizenship and Immigration Services (USCIS) San Fernando Valley Field Office (SFV);

2013 Speaker, 30th Anniversary of the Armenian Studies Program, CSUN, Northridge, CA;

2013 Guest Speaker, Kiwanis International, Northridge Chapter, Northridge, CA;

2014 Invited Speaker, *Toxicity of Heavy Metals and a Long-Term Impact of Irresponsible Mining on Public Health*, CSUN, Northridge, CA;

2014 Invited Speaker, *Antioxidants and Natural Supplements: Unraveling the Truth,* Pierce College, Woodland Hills, CA;

2014 Invited Speaker, *American Public's Perpetual Struggle Against Harmful Business Practices*, Public Advocacy Group Renaissance Armenia (via satellite), Yerevan;

2014 Speaker, *Why "Natural" Is Not Synonymous to "Good" and Harmless*," Vroman's Bookstore, Pasadena, CA.

2014 Invited Speaker, *Antioxidants, Supplements, and Cosmetics: Perils of Ignoring Science,* Regional High School Science Teachers' Conference, Ferrahian High School, Encino, CA;

2014 Invited Speaker, *Teaching Science in the 21st Century: Challenges and Solutions,* Regional High School Science Teachers' Conference, Ferrahian High School, Encino, CA;

REVIEWER / EVALUATOR / PANELIST / CONSULTANT / ADVISOR

Reviewer: National Science Foundation (NSF), American Chemical Society, Petroleum Research Fund (ACS PRF), *J. Amer. Chem. Soc.*, *Chem. Rev.*, *Org. Lett.*, *Tetrahedron*, *J. Organometal. Chem.*, *J. Cluster Sci.*, and *Canadian J. Chem.*; ARPA Institute, Student Innovation Competition; State Committee on Science and Technology, Armenia.

Panelist: National Science Foundation (NSF), California Writers' Club, San Fernando Valley, CA.

Consultant: Round Table Group, Washington, D.C.; National Academy of Sciences, Armenia; Consumer Advocacy Group, Los Angeles, CA; Renaissance Armenia Los Angeles, Los Angeles, CA.

National Registries: *2013-2014 Business Who's Who Registry*.

Scientific Advisory Board: American University of Armenia (AUA).

AFFILIATIONS

American Chemical Society; American Chemical Society, Organic Division; ACS Consulting Network; California Faculty Association (CFA); American Association of University Professors (AAUP); California Teachers Association (CTA); National Education Association (NEA); Service Employees International Union (SEIU); California Writers' Club - San Fernando Valley & West Hills Chapters; Independent Writers of Southern California (IWOSC); Institute for Community Health and Wellbeing (CSUN); Book Publicists of Southern California (BPSC); Union of Concerned Scientists (UCS); Public Citizen Foundation.

EXHIBIT B

PB93-185270

Evaluation of the Potential Carcinogenicity of Diaminotoluene (Mixed) (95-80-7)

Syracuse Research Corp., NY

Prepared for:

Environmental Protection Agency, Washington, DC

Jun 88

U.S. DEPARTMENT OF COMMERCE
National Technical Information Service
NTIS

PB93-185270

UNITED STATES
ENVIRONMENTAL PROTECTION
AGENCY

EPA/600/8-91/103
JUNE 1988
FINAL

RESEARCH AND
DEVELOPMENT

EVALUATION OF THE POTENTIAL CARCINOGENICITY OF DIAMINOTOLUENE (MIXED) (95-80-7)

IN SUPPORT OF REPORTABLE QUANTITY ADJUSTMENTS
PURSUANT TO CERCLA SECTION 102

PREPARED FOR
OFFICE OF EMERGENCY AND REMEDIAL RESPONSE
OFFICE OF SOLID WASTE AND EMERGENCY RESPONSE

PREPARED BY
CARCINOGEN ASSESSMENT GROUP
OFFICE OF HEALTH AND
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WASHINGTON, D.C. 20460

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4. TITLE AND SUBTITLE Evaluation of the Potential Carcinogenicity of Diaminotoluene (mixed) (95-80-7)	5. REPORT DATE June 1988
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Syracuse Research Corporation Environmental Monitoring & Services, Inc.	8. PERFORMING ORGANIZATION REPORT NO. 08CA-C-073-077
9. PERFORMING ORGANIZATION NAME AND ADDRESS Syracuse Research Corporation, Syracuse, NY Environmental Monitoring & Services, Inc. (now ABB Environmental Services, Inc.), Washington, DC	10. PROGRAM ELEMENT NO.
	11. CONTRACT/GRANT NO. 68-03-3112 68-03-3182
12. SPONSORING AGENCY NAME AND ADDRESS Office of Health and Environmental Assessment Carcinogen Assessment Group (RD-689) U.S. Environmental Protection Agency Washington, DC 20460	13. TYPE OF REPORT AND PERIOD COVERED
	14. SPONSORING AGENCY CODE EPA/600/021

15. SUPPLEMENTARY NOTES

16. ABSTRACT

Diaminotoluene is a probable human carcinogen, classified as weight-of-evidence Group B2 under the EPA Guidelines for Carcinogen Risk Assessment (U.S. EPA, 1986a). Evidence on potential carcinogenicity from animal studies is "Sufficient," and the evidence from human studies is "No Data." This evidence is based on the carcinogenic properties of the isomer 2,4-diaminotoluene.

The potency factor (F) for 2,4-diaminotoluene is estimated to be 23.2 $(mg/kg/day)^{-1}$, placing it in potency group 2 according to the CAG's methodology for evaluating potential carcinogens (U.S. EPA, 1986b).

Combining the weight-of-evidence group and the potency group, diaminotoluene is assigned a "MEDIUM" hazard ranking for the purposes of RQ adjustment, which assignment is applicable to all isomers of diaminotoluene.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT Release to Public	19. SECURITY CLASS (This Report) Unclassified	21. NO. OF PAGES 19
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PREFACE

This report summarizes and evaluates information on the potential carcinogenicity of a substance designated as hazardous under Section 101 (14) of the Comprehensive Environmental Response, Compensation and Liability Act of 1980 (CERCLA). Pertinent epidemiologic and toxicologic data were obtained through on-line searches and from hard-copy sources. On-line searches were extended as far back as the data bases would allow. Retrieval of historical data was accomplished through searches of hard-copy sources and bibliographies of relevant publications. Every attempt has been made to rely upon primary publications as opposed to data summaries or abstracts contained in secondary sources such as monographs, surveys, review articles, criteria documents, etc. The on-line data bases that were searched included CHEMLINE (National Library of Medicine (NLM)), RTECS (NLM), Toxicology Data Bank (NLM), TOXLINE (NLM), CANCERLINE (NLM), and Chemical Abstracts (DIALOG Information Services). Unpublished data were not used in this evaluation.

The Agency's Methodology for obtaining, evaluating, and ranking CERCLA potential carcinogens is described in the Technical Background Document to Support Rulemaking Pursuant to CERCLA Section 102, Volume 3, April 26, 1988 (EPA/600/8-89/053). This document revises the previous methodology document of 1986 according to the public comments received on the March 16, 1987 Notice of Proposed Rulemaking (52 FR 8140) The Methodology for Adjusting reportable quantities is described in the Technical Background Document to Support Rulemaking Pursuant to CERCLA Section 102, Volume 1, March, 1985, and is also summarized in Volume 2, August, 1986, and Volume 3, December, 1986. The EPA's Office of Emergency and Remedial Response (OERR) has considered this evaluation in adjusting reportable quantities pursuant to CERCLA Section 102. This report is consistent with the revised methodology. It draws largely on information supplied by the Syracuse Research Corporation in 1984 under EPA Contract No. 68-02-3112. Due to the amount of time elapsed between the original work performed by Syracuse Research Corporation and the present

effort to produce this document, Environmental Monitoring & Services, Inc., under EPA Contract No. 68-03-3182, has been involved in an extensive review of all the Syracuse documents. In some cases, this review involved updating the information provided but it was primarily a quality assurance effort. The present document is a result of this effort.

ABSTRACT

Diaminotoluene is a probable human carcinogen, classified as weight-of-evidence Group B2 under the EPA Guidelines for Carcinogen Risk Assessment (U.S. EPA, 1986a). Evidence on potential carcinogenicity from animal studies is "Sufficient," and the evidence from human studies is "No Data." This evidence is based on the carcinogenic properties of the isomer 2,4-diaminotoluene.

The potency factor (F) for 2,4-diaminotoluene is estimated to be 23.2 $(mg/kg/day)^{-1}$, placing it in potency group 2 according to the OAQ's methodology for evaluating potential carcinogens (U.S. EPA, 1986b).

Combining the weight-of-evidence group and the potency group, diaminotoluene is assigned a "MEDIUM" hazard ranking for the purposes of RQ adjustment, which assignment is applicable to all isomers of diaminotoluene.

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1.0 WEIGHT OF EVIDENCE

1.1 ANIMAL STUDIES

Diaminotoluene (Waste No. U221) comprises six isomers, the 2,4-isomer being the most important industrially. Nearly 300M pounds are produced annually. The 2,4-diaminotoluene isomer is an intermediate in the synthesis of polyurethane. Therefore, for purposes of hazard ranking, the bioassay studies of this isomer will be used as a model for the U221 waste.

Long-term dietary administration of 2,4-diaminotoluene induced dose-related hepatocellular carcinomas in male rats (Ito et al., 1969) and female mice (NCI, 1979). Ito et al., (1969) found multiple metastases in many of the treated rats, but primary neoplasms were not observed in organs other than the liver. NCI (1979) also found that the combined incidence of hepatocellular carcinomas and neoplastic nodules was significantly elevated in male rats when compared with untreated controls; the significance of the occurrence of the liver tumors in the male and female rats is supported by a high incidence of associated non-neoplastic liver lesions in the dosed groups, and by low incidences of liver tumors in historical control rats. The hepatocarcinogenic potential of 2,4-diaminotoluene is further supported by the unpublished results of 1-year studies at the DuPont Haskell laboratories (Occupational Health and Safety Letter, 1975), and 2-year studies that were conducted by E.K. Weisburger et al. (n.d.). In the Weisburger study, "low incidences" of liver tumors were reportedly observed in CD-1 (Sprague-Dawley) rats that were administered diets containing 2,4-diaminotoluene dihydrochloride at doses of 500 to 1000 ppm for 4 months, and subsequently, 250 or 500 ppm for 14 months, and in NmM/ICR mice that were dosed at levels of 500 or 1000 ppm for 18 months.

When 2,4-diaminotoluene was administered to 20 rats of mixed strain and sex by weekly subcutaneous injection, 11 died within 8 months without marked tissue changes (Umeda, 1955). All of the 9 surviving rats, which had received 29 to 44 weekly injections, developed subcutaneous sarcomas.

Chronic dietary administration of 2,4-diaminotoluene was also associated with the development of subcutaneous fibromas in male rats, mammary gland carcinomas or adenomas in female rats, and lymphomas in female mice (NCI, 1979).

The highest incidence of cancer in any of the animal studies was mammary gland tumors (adenoma or carcinoma) in F 344 female rats. Data reported in the one-year DuPont study also support the conclusion that the mammary gland is the site of increased carcinoma formation in female rats. The NCI study was chosen for estimating the potency factor.

1.2 HUMAN STUDIES

Pertinent data regarding the carcinogenic effects of exposure to 2,4-diaminotoluene in humans were not located in the available literature.

1.3 WEIGHT-OF-EVIDENCE ASSESSMENT

The induction of hepatocellular carcinomas in several strains of rats and mice following oral exposure, and supporting evidence that oral exposure also induces tumors at sites other than the liver and that local sarcomas are produced by repeated subcutaneous injection, provide sufficient evidence that 2,4-diaminotoluene is an animal carcinogen. There are no human case reports or epidemiological studies on 2,4-diaminotoluene. Thus, using the EPA Guidelines for Carcinogen Risk Assessment (U.S. EPA, 1986a) for evaluating the overall weight of evidence to humans, 2,4-diaminotoluene is most appropriately classified as a Group B2 chemical. The appendix contains summaries of the significant human and/or animal studies cited in this review.

2.0 POTENCY

The potency factor (F) for 2,4-diaminotoluene is estimated to be 23.2 $(mg/kg/day)^{-1}$, placing it in potency group 2 under the CAG's methodology for evaluating potential carcinogens (U.S. EPA, 1986b). Table 2-1 contains data from the selected study used to derive the potency factor for 2,4-diaminotoluene.

Table 2-1. Derivation of Potency Factor(F) Agent: 2,4-Diaminotoluene

REFERENCE:	NCI, 1979		
EXPOSURE ROUTE:	oral		
SPECIES:	rats		
STRAIN:	F 344		
SEX:	F		
VEHICLE OR PHYSICAL STATE:	diet		
BODY WEIGHT:[a]	0.175 kg (high dose), 0.22 kg (low dose), 0.275 kg (control)		
DURATION OF TREATMENT:	588 days	721 days	721 days
DURATION OF STUDY:	588 days	721 days	721 days
LIFESPAN OF ANIMAL:[b]	728 days	728 days	728 days
TARGET ORGAN:	mammary gland		
TUMOR TYPE:	adenoma		
EXPERIMENTAL DOSES/ EXPOSURE:	171 ppm	79 ppm	0 ppm
TRANSFORMED DOSES:[c] (mg/kg/day)	8.55	3.95	0
TUMOR INCIDENCE:	42/50	38/50	1/20
ANIMAL POTENCY: $(mg/kg/day)^{-1}$	2.48		
HUMAN POTENCY:[d] $(mg/kg/day)^{-1}$	23.2		

[a] Reported

[b] Assumed

[c] to derive the transformed dose from the experimental dose data: experimental dose (ppm) x 0.05 (fraction of species body weight consumed as food per day) x duration of treatment (days)/duration of study (days).

[d] human potency = animal potency x $(70\ kg/0.223\ kg)^{1/3}$, where 0.223 kg is the average body weight of the rats, x $(728\ days/645.5\ days)^{3}$ to adjust for the short study duration, where 645.5 days is the average of the three study durations listed above.

3.0 HAZARD RANKING

Based on the weight-of-evidence Group B2 and the potency factor (F) of 23.2 (mg/kg/day)-1; diaminotoluene receives a hazard ranking of "MEDIUM." This hazard ranking, which is based on the isomer 2,4-diaminotoluene, is applicable to all isomers of diaminotoluene.

4.0 REFERENCES

Ito, N., Y. Hiasa, Y. Konishi and M. Marugami, 1969. The Development of Carcinoma in Liver of Rats Treated with Meta-Toluylenediamine and the Synergistic and Antagonistic Effects with Other Chemicals. Cancer Res. 29: 1137-1145.

NCI (National Cancer Institute), 1979. Bioassay of 2,4-Diaminotoluene for Possible Carcinogenicity. NCI Carcinogens Technical Report Series No. 162. DHEW Publication No. (NIH) 79-1718.

Occupational Health and Safety Letter, May 22, 1975. (Cited in NCI, 1979).

Umeda, M., 1955. Production of Rat Sarcoma by Injections of Propylene Glycol Solution of m-Toluylenediamine. Gann. 46: 597-603.

U.S. EPA (Environmental Protection Agency), 1986a. Guidelines for Carcinogen Risk Assessment, 51 FR 33992-34003, September 24, 1986.

U.S. EPA (Environmental Protection Agency), 1986b. Methodology for Evaluating Potential Carcinogenicity in Support of Reportable Quantity Adjustments Pursuant to CERCLA Section 102, OHEA-C-073, December 1986. Available from CERCLA Docket 102RQ-273C. The public docket for RQ rulemaking is located in room M2427, U.S. Environmental Protection Agency, 401 M Street, SW, Washington, DC 20460. It is available for inspection Monday through Friday excluding Federal holidays, between the hours of 9:00 a.m. and 4:00 p.m.

U.S. EPA (Environmental Protection Agency), 1988. Technical Background Document to Support Rulemaking Pursuant to CERCLA Section 102, Volume 3, Draft, Appendix A, April 26, 1988.

Weisburger, E.K., A.B. Ruefield, F. Homburger, J.H. Weisburger, E. Boger, G.G. Van Dongen and K.C. Chu. (n.d.). Testing of 21 Environmental Aromatic Amines or Derivatives for Long-Term Toxicity or Carcinogenicity. In press. National Cancer Institute, Bethesda, Maryland. (Cited in NCI, 1979).

APPENDIX

SUMMARY OF SIGNIFICANT HUMAN AND/OR ANIMAL STUDIES

Table A. Animal

Agent: 2, 4-Diaminotoluene

Reference: Ito et al., 1969

Exposure Route	Species/ Strain	Sex	Dose or Exposure	Duration of Treatment	Duration of Study	Purity of Compound	Vehicle or Physical State	Target Organ	Tumor Type	Tumor Incidence (P value)
Experiment 1										
o	rats/ Wistar	M	0.1%	33 weeks[a]	33 weeks[a]	NR	diet	liver	hepatocellular carcinoma	9/9[b]
o	rats/ Wistar	M	0.86%	35 weeks[a]	35 weeks[a]	NR	diet	liver	hepatocellular carcinoma	7/11[c]
o	rats/ Wistar	M	0%	56 weeks(a)	56 weeks[a]	NR	diet	liver	hepatocellular carcinoma	0/6[d]
Experiment 2										
o	rats/ Wistar	M	0.1%	32 weeks[a]	32 weeks[a]	NR	diet	liver	hepatocellular carcinoma	6/6
o	rats/ Wistar	M	0%	56 weeks[a]	56 weeks[a]	NR	diet	liver	hepatocellular carcinoma	0/6

QUALITY OF EVIDENCE

Strengths of Study: Two dose levels were tested and multiple experiments were performed. The compound was administered by a natural route of exposure.

Weaknesses of Study: Small numbers of treatment and control rats were used.

Overall Adequacy: Adequate.

[a] Average duration of survival (rats were observed until death or until they became moribund).

[b] 9/12 rats survived an average of 33 weeks. The 9 survivors had multiple hepatocellular carcinomas, and 6 of the 9 survivors had multiple metastases in the lymph nodes, omentum, lungs, or epididymis.

[c] 11/12 rats survived an average of 35 weeks. Seven of the 11 survivors had multiple hepatocellular carcinomas with metastases in the lymph nodes and omentum.

[d] 6/6 rats survived 56 weeks of control treatment. No evidence of non-neoplastic liver damage.

Table K. Animal

Agent: 2,4-Diaminotoluene

Reference: NCI, 1979

Exposure Route	Species/ Strain	Sex	Dose or Exposure	Duration of Treatment	Duration of Study	Purity of Compound	Vehicle or Physical State	Target Organ	Tumor Type	Tumor Incidence (P value)[d]
o	rats/ Fischer	M	176 ppm[a]	79 weeks[c]	79 weeks	>99.9%	diet	liver	hepatocellular carcinomas or neoplastic nodules	10/50[e] (P=0.026)
o	rats/ Fischer	M	176 ppm[a]	79 weeks[c]	79 weeks	>99.9%	diet	liver	hepatocellular carcinomas or neoplastic nodules	6/50 5/50
o	rats/ Fischer	M	176 ppm[a]	79 weeks[c]	79 weeks	>99.9%	diet	sc tissue	fibromas	19/50[f] (P=0.004)
o	rats/ Fischer	M	79 ppm[b]	103 weeks	103 weeks	>99.9%	diet	liver	hepatocellular carcinomas or neoplastic nodules	5/49[g] (NS)
o	rats/ Fischer	M	79 ppm[a]	103 weeks	103 weeks	>99.9%	diet	liver	hepatocellular carcinomas or neoplastic nodules	3/49 2/49
o	rats/ Fischer	M	79 ppm[a]	103 weeks	103 weeks	>99.9%	diet	sc tissue	fibromas	15/30[f] (P=0.020)
c	rats/ Fischer	M	0 ppm	103 weeks	103 weeks	NA	NA	liver	hepatocellular carcinomas or neoplastic nodules	0/20
o	rats/ Fischer	M	0 ppm	103 weeks	103 weeks	NA	diet	sc tissue	fibroma	0/20
o	rats/ Fischer	F	171 ppm[b]	84 weeks[c]	84 weeks	>99.9%	diet	liver	hepatocellular carcinomas or neoplastic nodules	6/49[g] (NS)

Table A. Animal

Reference: NCI, 1979 (cont.)

Exposure Route	Species/ Strain	Sex	Dose or Exposure	Duration of Treatment	Duration of Study	Purity of Compound	Vehicle or Physical State	Target Organ	Tumor Type	Tumor Incidence (P value)[d]
o	rats/ Fischer	F	171 ppm[b]	84 weeks[c]	84 weeks	>99.9%	diet	liver	hepatocellular carcinomas or neoplastic nodules	3/49 5/49
o	rats/ Fischer	F	171 ppm[b]	84 weeks[c]	84 weeks	>99.9%	diet	mammary gland	carcinomas or adenomas	42/50 (P<0.001)
o	rats/ Fischer	F	171 ppm[b]	84 weeks[c]	84 weeks	>99.9%	diet	mammary gland	carcinomas	8/50
o	rats/ Fischer	F	171 ppm[b]	84 weeks[c]	84 weeks	>99.9%	diet	mammary gland	fibroadenomas	29/50
o	rats/ Fischer	F	171 ppm[b]	84 weeks[c]	84 weeks	>99.9%	diet	mammary gland	all other adenomas	18/50
o	rats/ Fischer	F	79 ppm[b]	103 weeks	103 weeks	>99.9%	diet	liver	hepatocellular carcinomas or neoplastic nodules	0/50[e] (NS)
o	rats/ Fischer	F	79 ppm[b]	103 weeks	103 weeks	>99.9%	diet	mammary gland	carcinomas or adenomas	38/50 (P<0.001)
o	rats/ Fischer	F	79 ppm[b]	103 weeks	103 weeks	>99.9%	diet	mammary gland	carcinomas	9/50
o	rats/ Fischer	F	79 ppm[b]	103 weeks	103 weeks	>99.9%	diet	mammary gland	fibroadenomas	26/50
o	rats/ Fischer	F	79 ppm[b]	103 weeks	103 weeks	>99.9%	diet	mammary gland	all other adenomas	13/50

A-4

Table A. Animal

Reference: NCI, 1979 (cont.)

Exposure Route	Species/ Strain	Sex	Dose or Exposure	Duration of Treatment	Duration of Study	Purity of Compound	Vehicle or Physical State	Target Organ	Tumor Type	Tumor Incidence (P value)[d]
o	rats/ Fischer	F	0 ppm	103 weeks	103 weeks	NA	NA	liver	hepatocellular carcinomas or neoplastic nodules	0/20
o	rats/ Fischer	F	0 ppm	103 weeks	103 weeks	NA	NA	mammary gland	carcinomas or adenomas	1/20
o	rats/ Fischer	F	0 ppm	103 weeks	103 weeks	NA	NA	mammary gland	carcinomas	0/20
o	rats/ Fischer	F	0 ppm	103 weeks	103 weeks	NA	NA	mammary gland	fibroadenoma	1/20
o	rats/ Fischer	F	0 ppm	103 weeks	103 weeks	NA	NA	mammary gland	all other adenomas	0/20
c	mice/ B6C3F1	M	200 ppm	101 weeks	101 weeks	>99.9%	diet	liver	hepatocellular carcinoma	13/49 (NS)
o	mice/ B6C3F1	M	100 ppm	101 weeks	101 weeks	>99.9%	diet	liver	hepatocellular carcinoma	17/50 (NS)
o	mice/ B6C3F1	M	0 ppm	101 weeks	101 weeks	NA	NA	liver	hepatocellular carcinoma	5/20
o	mice/ B6C3F1	F	200 ppm	101 weeks	101 weeks	>99.9%	diet	liver	hepatocellular carcinoma	18/48[f] (P=0.001)
o	mice/ B6C3F1	F	200 ppm	101 weeks	101 weeks	>99.9%	diet	hemato-poietic system	lymphomas or lymphocyte leukemia	11/48 (NS)
o	mice/ B6C3F1	F	100 ppm	101 weeks	101 weeks	>99.9%	diet	liver	hepatocellular carcinoma	[illegible][f] (P=0.057)

A-9

Table A. Animal

Reference: NCI, 1979 (cont.)

Exposure Route	Species/ Strain	Sex	Dose or Exposure	Duration of Treatment	Duration of Study	Purity of Compound	Vehicle or Physical State	Target Organ	Tumor Type	Tumor Incidence (P value)[d]
o	mice/ B6C3F1	F	100 ppm	101 weeks	101 weeks	>99.9%	diet	hemato-poietic system	lymphomas or lymphocyte leukemia	29/47 (P<0.001)
o	mice/ B6C3F1	F	0 ppm	101 weeks	101 weeks	NA	NA	liver	hepatocellular carcinoma	0/19
o	mice/ B6C3F1	F	0 ppm	101 weeks	101 weeks	NA	NA	hemato-poietic system	lymphomas or lymphocyte leukemia	2/19

QUALITY OF EVIDENCE

Strengths of Study: The compound was tested by a natural route of administration in two species. Two dose levels per species were employed, and the animals were exposed for a significant portion of their lifespan. Comprehensive histopathologic examinations were performed.

Weaknesses of Study: Early deaths may have reduced the incidences of the tumor in the high-dose groups.

Overall Adequacy: Adequate

[a] Time-weighted average dose. The compound was administered at levels of 250 ppm for 40 weeks and subsequently 100 ppm for 39 weeks (high dose), and 125 ppm for 40 weeks and subsequently 50 ppm for 63 weeks (low dose).

[b] Time-weighted average dose. The compound was administered at levels of 250 ppm for 40 weeks and subsequently 100 ppm for 44 weeks (high dose), and 125 ppm for 48 weeks and subsequently 50 ppm for 63 weeks (low dose).

[c] Administration of the diet was terminated early due to treatment-related morbidity.

[d] Fischer exact test. NS = Not Significant

[e-i] Significant Cochran-Armitage test for positive dose-related trend: (P=0.614)[e], (P=0.009)[f], (P=0.008)[g], (P=0.007)[h], (P=0.002)[i]

NA = Not applicable

EXHIBIT C

Proc. Nat. Acad. Sci. USA
Vol. 72, No. 6, pp. 2423–2427, June 1975

Hair Dyes Are Mutagenic: Identification of a Variety of Mutagenic Ingredients

(hair dyes as possible human carcinogens and mutagens/2,4-diaminoanisole/2,4-diaminotoluene)

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Contributed by Bruce N. Ames, March 13, 1975

ABSTRACT **We have previously described a sensitive bacterial test for detecting carcinogens as mutagens. We show here that 89% (150/169) of commercial oxidative-type (hydrogen peroxide) hair dye formulations are mutagenic in this test. Of the 18 components of these hair dyes, nine show various degrees of mutagenicity: 2,4-diaminoanisole, 4-nitro-*o*-phenylenediamine, 2-nitro-*p*-phenylenediamine, 2,5-diaminoanisole, 2-amino-5-nitrophenol, *m*-phenylenediamine, *o*-phenylenediamine, 2-amino-4-nitrophenol, and 2,5-diaminotoluene. Three hair dye components (*p*-phenylenediamine, 2,5-diaminotoluene, and 2,5-diaminoanisole) become strongly mutagenic after oxidation by H_2O_2: the mutagenic product of *p*-phenylenediamine is identified as the known trimer, Bandrowski's base. 2,4-Diaminotoluene, a hair dye component until recently, is also shown to be mutagenic: this compound has been shown to be a carcinogen in rats, and is used in large amounts in the polyurethane foam industry. About 20,000,000 people (mostly women) dye their hair in the U.S. and the hazard could be considerable if these chemicals are actually mutagenic and carcinogenic in humans.**

We have previously described a very sensitive and simple bacterial test for detecting chemical mutagens (1–3). The compounds are tested on petri plates with specially constructed mutants of *Salmonella typhimurium* as tester strains. Several tester strains were selected for sensitivity and specificity in being reverted from a histidine requirement back to prototrophy by a variety of mutagens. One strain (TA1535) detects mutagens causing base-pair substitutions and two (TA1537 and TA1538) detect various kinds of frameshift mutagens. In addition to the histidine mutation, we have added to each tester strain two additional mutations that greatly increase its sensitivity to mutagens: one causes loss of the excision repair system and the other loss of the lipopolysaccharide barrier that coats the surface of the bacteria (2).

A large number of carcinogens have been shown to be mutagens which cause base-pair substitutions in our test (1–3). Other carcinogens with aromatic rings have been shown to cause frameshift mutations (2–6). We have shown that by adding a microsomal activation system of rat (or human autopsy) liver to the petri plates, a wide variety of carcinogens can be detected as mutagens after they have been metabolized to their active forms (refs. 3, 7–9; and unpublished data). Thus, an important aspect of mammalian metabolism can be duplicated in an *in vitro* test.

The present work stems from a biochemistry class experiment in which hundreds of commercial products were tested for mutagenicity. Only two products were found to be mutagenic: cigarette smoke tar (8) and an oxidative-type hair dye. Since 20,000,000 people (10), mainly women, dye their hair in the U.S. we have made a detailed study of hair dye mutagenicity.

The two main types of hair dyes are the "semi-permanent" or direct color dyes and the "permanent" or oxidative-type dyes in which H_2O_2 is used to oxidize aromatic diamines with the production of larger colored molecules which are trapped in the hair shaft. We have concentrated on the oxidative-type dyes since they account for about 75% (11) of the $250,000,000/year hair dye market. The oxidative dye package consists of one bottle containing a mixture of aromatic amines, aromatic nitro derivatives, and phenols in a vehicle liquid (12, 13), and a bottle of H_2O_2 with which it is mixed immediately before use.

RESULTS

We first tested about 30 oxidative-type hair dye formulations for reversion of our standard tester strains. Almost all of these were mutagenic when tested on strain TA1538, indicating the production of frameshift-type mutations. Strain TA1535 was not reverted, indicating a lack of base-pair substitution mutations; the other frameshift tester strain, TA1537, with a different specificity also was not reverted. We have, therefore, used strain TA1538 to test 169 different oxidative-type dye formulations; these were all the products we could find in two local drug stores. Each hair dye preparation has been tested both before and after mixing with H_2O_2. A few typical examples are shown in Table 1 and Fig. 1. Almost all of the dyes were quite mutagenic: 150 of 169 tested or 89%, and almost all retained activity after peroxide treatment. We tested product lines of eight different companies: Clairol Inc. (Shampoo-in Toner Naturally Blonde, Miss Clairol Creme Formula Hair Color Bath, Balsam Color Conditioning Shampoo-in Haircolor, Creme Toner Salon Formula Oil Shampoo Tint, Born Blonde Lotion Toner, True Brunette The True-Color Hair Color, Nice'n Easy); John H. Breck, Inc. (Beautiful Hair Breck Hair Color); Revlon (Revlon Colorsilk); Alberto-Culver Co. (For Brunettes Only); Tussy Cosmetics, Inc. (Ogilvie Shampoo-in Conditioning Hair Color); Cosmair, Inc. (L'Oreal Preference Permanent Creme-in Haircolor, L'Oreal Excellence Permanent Hair Color); The Gillette Co. (Toni Shampoo-easy Haircoloring for Innocent Color); Roux Laboratories Inc. (Roux fancitone creme Hair Tint). Mutagenic hair dyes were found in every hair dye product line tested. Table 1 shows that some of the dyes were mutagenic directly, without microsomal activation, whereas others required microsomal activation (addition of S-9 mix). In addition, some of the dyes were not mutagenic until they were mixed with H_2O_2. Thus, there must be at least three different ingredients responsible for mutagenicity.

Eighteen different chemicals are used in oxidative hair dyes in the U.S., in addition to H_2O_2 and those in the vehicle liquid (C. Burnett, personal communication), and industry

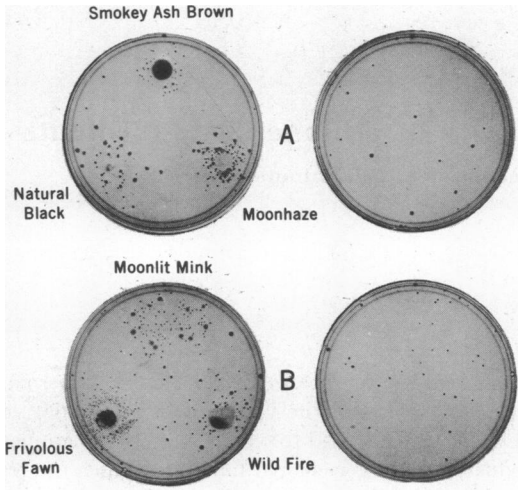

FIG. 1. Spot tests on petri plates showing the mutagenicity of various hair dyes on strain TA1538: Moonhaze no. 32 (Miss Clairol), Moonlit Mink no. 360 (Clairol Born Blonde), Wild Fire no. 32 Roux fancitone) (all tested without peroxide), and Frivolous Fawn no. 23 (Roux fancitone) (mixed with H_2O_2 as per instructions). The procedure was as described in Table 1. Control plates without hair dyes are on the right. Plates B, but not A, contain liver microsomes (S-9 Mix). Smokey Ash Brown no. 775 and Natural Black no. 83 (Clairol Loving Care) are semi-permanent, non-oxidative type dyes (see *text*).

has kindly supplied them to us. The results of mutagenicity tests are shown in Fig. 2. Nine of the hair dye chemicals tested are mutagenic and their potencies in reverting strain TA1538 cover a 200-fold range. We also show the mutagenicity

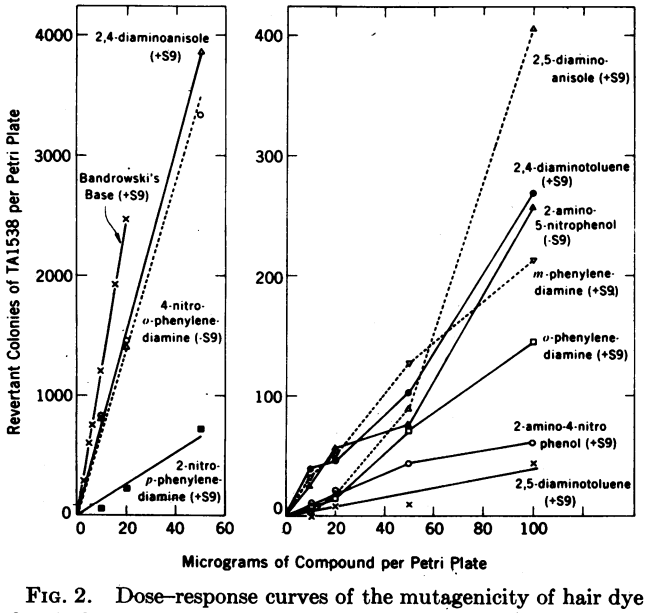

FIG. 2. Dose–response curves of the mutagenicity of hair dye chemicals for tester strain TA1538. Reversion was determined on petri plates by incorporating the chemical mutagen, bacteria, and microsomal activation system where indicated (see legend to Table 1) directly into the top agar (3). Revertants were counted after 48 hr at 37° and spontaneous revertant controls (<40 colonies) have been subtracted. All of the dimethylsulfoxide solutions of the chemicals were found to be sterile. 2,4-Diaminotoluene was the HCl salt and both diaminoanisole compounds were the sulfate salts: all were used as such.

TABLE 1. *Mutagenic activity of hair dyes in reverting strain TA1538*

	Revertant colonies per spot*			
	Hair dye†		Mixed with H_2O_2‡	
	−S9	+S9	−S9	+S9
Medium Golden Brown no. 612 (Clairol Balsam Color)	555	176	294	173
Natural Dark Brown no. 120 (Clairol Nice'N Easy)	131	151	84	244
Pale Ash Blonde no. 120 (Toni Innocent Color)	2	69	0	35
Medium Brown no. 17 (Breck Shampoo-in Hair Color)	0	3	0	267
Light Golden Brown no. 7 (Breck Shampoo-in Hair Color)	4	0	0	3

* Control values have been subtracted (<12 colonies). The dyes were found to be sterile. Values greater than 10 colonies over the control value were interpreted as positive.

† A drop (about 40 μl) of a freshly opened bottle of hair color was spotted on a petri plate containing tester strain TA1538 in a thin agar overlay (3) and, where indicated, S-9 Mix (3) containing rat liver homogenate from rats induced with Aroclor 1254 was added (8).

‡ The hair dye was mixed (in a sterile tube) with an equal volume of hydrogen peroxide supplied by the manufacturer, as per instructions. Since the drop size was the same (40 μl), the numbers should be doubled to make them comparable to the unmixed dye.

of 2,4-diaminotoluene, a chemical that was removed from hair dyes in 1971 after it was shown to be a carcinogen when fed to rats (14). The hair dye component that was most active in reverting TA1538 is the very closely related compound 2,4-diaminoanisole (a CH_3O– group is substituted for a CH_3– group) which we find is about 30 times more active than the toluene derivative. Addition of human (3) or rat liver microsomes gives very similar results with these two compounds. We have previously shown that many carcinogenic aromatic amines or aromatic nitro compounds revert strain TA1538 (3, 9). For example, we have repeated our dose-response curve for benzidine·2HCl (3), an aromatic diamine which is a carcinogen for humans, and find it similar in activity to *m*-phenylenediamine. The dose–response curves have been duplicated with the purest chemicals available commercially (Aldrich or Eastman). Nevertheless, many of these compounds are easily oxidized in air and it is quite possible that these (or other) impurities could account for the mutagenicity of some of the less active compounds (see below).

The aromatic amines (Fig. 2) all require activation with microsomes (S-9) for mutagenesis; this is characteristic of a variety of aromatic amine carcinogens (3, 5, 7–9). Nitro carcinogens, such as 2-nitrofluorene, mutate the bacteria without microsomal activation (2, 9) because the bacteria contain nitro reductases which activate them (as do mammalian cells). Thus, 4-nitro-*o*-phenylenediamine could account for most of the mutagenicity of those hair dyes that do not require microsomal activation. [It is added to hair dyes as a direct color and is not appreciably oxidized by H_2O_2 (12, 15)]. In one case, Natural Dark Brown no. 120, the mutagenic activity measured without microsomes can be exactly

accounted for by its content of this chemical (0.25 mg/ml). The mutagenic activity of 2-nitro-*p*-phenylenediamine and 2-amino-4-nitrophenol is much greater if microsomes are added, and since these compounds also have amino groups, the activation presumably involves an amino oxidation in addition to, or instead of, a nitro reduction.

The following components of hair dyes showed no mutagenic activity in a spot test (about 1 mg) against strains TA97, TA98, TA100, and TA1538 (2, 9): *p*-aminophenol, *m*-aminophenol, resorcinol, 1-naphthol, 4-chlororesorcinol, *p*-aminodiphenylamine, proprietary *p*-phenylenediamine derivative, *N*-methyl-*p*-aminophenol, and *p*-phenylenediamine. Neither the hair dye vehicle, nor the H_2O_2, nor their mixture, were found to revert TA1538 at the level tested (about 40 μl in a spot test).

There is a marked increase in mutagenicity of certain hair dyes upon mixing with H_2O_2 and in order to identify some of the responsible ingredients, we have treated all of the individual nonmutagenic components of the hair dyes with H_2O_2. After oxidation, *p*-phenylenediamine gives a very strong mutagenic response with strain TA1538 when S-9 is present. This reaction is known to yield mainly Bandrowski's base (12, 13), a trimer of *p*-phenylenediamine. We have prepared crystalline Bandrowski's base (16) and have also obtained a sample from J. F. Corbett. Although this compound is entremely active in reverting strain TA1538 (Fig. 2), it is thought to be only a very minor product in a typical hair dye oxidation (17). We have also examined the other *para* diamino compounds, 2,5-diaminotoluene and 2,5-diaminoanisole, which are weakly active (Fig. 2). Each of these (at 0.25 mg/ml or 10 mg/ml in hair dye vehicle) was mixed with peroxide and spotted on a petri plate with S-9, as described in Table 1. After oxidation, we found over a 40-fold increase in revertants for both compounds. A myriad of products is produced on oxidation when a mixture of about eight amines and phenols is present in hair dye (12, 13). The main products are open, conjugated 2- and 3-ring indamine and indoaniline dyes. Some minor products with 3-fused rings, such as aminophenazines and aminophenoxazinones, are formed as well (12, 13).

Although we have examined oxidative-type dyes in most detail, 25 different semipermanent-type dyes were also tested. Most of these were mutagenic as well (see Smokey Ash Brown and Natural Black in Fig. 1). The semi-permanent dyes contain a mixture of direct colors (2.4–6 g per dyeing) (15). A very large number of chemicals are used (12, 15), including the mutagenic 4-nitro-*o*-phenylenediamine and such polycyclic substances as aminoanthraquinones.

DISCUSSION

We (J. McCann and B.N.A.) are presently compiling the results of the *Salmonella*/mammalian microsome mutagenesis test on the hundreds of carcinogens and non-carcinogens tested in this laboratory and many others. The results compiled so far indicate that about 85% of carcinogens tested are detected as mutagens. These include a wide variety of aromatic amine carcinogens, aromatic nitro carcinogens, azo dyes, aflatoxin, and polycyclic hydrocarbons (3, 9). Even considering the uncertainty in classifying compounds as *non-carcinogens* (because of limited data from many cancer tests on rodents), relatively few (< 10%) of the compounds classified as non-carcinogens (many close relatives of carcinogens have been tested) have shown mutagenic activity. In addition, it has been our experience that extremely few chemicals in general show mutagenic activity: this includes hundreds of substances tested in the student biochemistry laboratories, complex mixtures such as urine (7), and many hundreds of chemicals about whose carcinogenicity nothing is known.

Despite the possibility that particular classes of bacterial mutagens may be found that are not carcinogenic, it appears that each of the hair dye compounds we have found to be mutagenic has a high probability of proving to be a carcinogen. This conclusion is in agreement with our detection as mutagens of the somewhat related aromatic amines benzidine, 4-aminobiphenyl and β-naphthylamine (3), which are carcinogens for humans, and 2,4-diaminotoluene, which is a carcinogen in rats (14). In addition to a potential carcinogenicity, many of the compounds that are mutagenic for bacteria may be hazardous to humans by causing mutations in the germ line (18).

We would like to emphasize that our test measures mutagenesis at specific base sequences, e.g., the -C-G-C-G-C-G-C-G sequence (6) in strain TA1538, and does not necessarily indicate overall mutagenic potential. Attempts to make *quantitative* correlations between the amount of activity in our test and carcinogenic or mutagenic potency for humans is certainly premature, in any case.

Because a large fraction of the U.S. population uses hair dyes, we feel it is important to discuss the evidence concerning human absorption of hair dye components, the carcinogenicity of the substances, and the possible human risk involved. As an aid in the discussion, we note that the three *meta* diamines are the series *m*-phenylenediamine, 2,4-diaminotoluene, and 2,4-diaminoanisole, and that the three *para* diamines are *p*-phenylenediamine, 2,5-diaminotoluene, and 2,5-diaminoanisole: both a *para* compound and a coupler, such as a *meta* compound or resorcinol, are needed for proper dye formation on oxidation (12, 13).

Absorption of Hair Dyes in Humans. Surprisingly little has been published about the absorption of the various components of hair dyes through the skin of the human scalp. Kiese *et al.* (19) have published an excellent study, though only on one of the amines in oxidative hair dyes. They examined the urine of five volunteers who had used a simplified hair dye preparation consisting of the liquid vehicle containing detergents, 1.38 g of 2,5-diaminotoluene (the *para* compound), resorcinol (the coupler), and H_2O_2. They found that the diacetyl derivative of the 2,5-diaminotoluene was excreted in the urine for 2 days after hair dyeing, the average amount being 3.66 mg per person. From a measurement of the recovery factor (they injected the same volunteers with 2,5-diaminotoluene and determined that 48% appeared in the urine as the diacetyl derivative), they calculated that, at a minimum 4.6 mg of 2,5-diaminotoluene must have been absorbed during hair dyeing; this was 0.33% of the original 1.38 g (2.5 g of the H_2SO_4 salt) applied. The percentage of free diamine absorbed was probably much more than 0.33% since only a small portion of the 2,5-diaminotoluene applied to the scalp would not react with hydrogen peroxide and be present as free diamine. In addition to the 4.6 mg of 2,5-diaminotoluene absorbed, its reaction product with resorcinol, an

indoaniline dye (13), and other products were probably absorbed and metabolized, but the methods of Kiese *et al.* were only designed for determining the urinary metabolite diacetyl 2,5-diaminotoluene. It is possible that some of the highly mutagenic oxidation product mentioned previously was formed and absorbed to some extent. Facile absorption of more complex amine molecules is also indicated by experiments with dogs (20) showing that Bandrowski's base is absorbed through the skin. Moreover, many aromatic amines, and diamines such as benzidine, are absorbed well through human skin (21, 22). Thus, we believe it is reasonable to estimate that a woman undergoing one hair dyeing (with about 4 g of amines) could absorb as much as 40 mg (1%) of hair dye chemicals (precursors, products, and side products) through the scalp.

Carcinogenicity Studies of the meta Diamines: 2,4-Diaminotoluene, 2,4-Diaminoanisole, m-Phenylenediamine. 2,4-Diaminotoluene was a component of hair dyes until 1971. It was used at a level of 60 mg per dyeing (1 mg/kg) (C. Burnett, personal communication). This compound is also of unusual interest because of its widespread industrial use (U.S. 167 million lbs. in 1972) as a precursor of the toluene diisocyanate monomer of the polyurethane foam industry. Ito *et al.* (14) reported that rats fed 2,4-diaminotoluene at 0.06% and 0.1% of the diet showed 63% (7/11) and 100% (15/15) liver tumors, respectively. This followed up a much earlier, but less definitive study by Umeda (23) showing that its subcutaneous injection in rats caused 100% (9/9) sarcomas: 56 mg total dose (240 mg/kg). We have been informed that the National Cancer Institute studies on the three chemicals, by feeding to both rats and mice, have not yet been completed. (U. Saffiotti, personal communication).

Two carcinogenicity studies involving topical application to the skin of rodents of hair dye mixtures containing the above three chemicals, have been sponsored by cosmetic industry groups. Topical studies approximate more closely the actual conditions of use and, more importantly, test the mixture of compounds produced in a hair dye oxidation not just the ingredients. The two studies appear to us, however, to be quite inadequate for the purpose of showing safety for hair dye users and we therefore discuss them briefly. Kinkel and Holzmann (24), on behalf of German hair dye firms, did a long term ($2^1/_2$ year) study on carcinogenicity of a hair dye mixture containing 2,4-diaminoanisole, 2,5-diaminotoluene, resorcinol, vehicle, detergent, and peroxide, which were applied topically to the back of rats. They used no positive control. The experimental and control animals showed no statistically significant difference in type and incidence of tumors. For example, 28% female rats with cancer (14/50) in those treated with 2,4-diaminoanisole (approximately 7 mg/kg applied twice weekly for 2 years) as compared to 21% (26/125) not receiving this substance, is 7% excess of rats with cancer, but this can be attributable to chance. This experiment, therefore, could not have detected a chemical that increased cancer by 5%. However, a 5% excess of cancers in humans would be 1 million people out of the 20,000,000 at risk. Thus, it obviously is necessary to perform experiments with rats in which the variation attributable to chance is well below the 7% value. The statistical limitations of small numbers of animals are usually overcome by using much higher doses of the chemical in the animal than the human is exposed to: this wasn't done in these two studies. The amount of 2,4-diaminoanisole currently used in hair dyes is about 600 mg/dyeing (10 mg/kg) (C. Burnett, personal communication). Thus, if a woman dyed her hair monthly for about 2 years (equivalent dose based on the surface area comparison*) or for about 12 years (based on the mg/kg comparison*) she could* have been exposed to a cumulative applied dose equivalent to that of the rats above.

A similar type of carcinogenicity study (11), also reported as negative, has been done by dyeing mice topically (50 of each sex per test group) using three formulations, each with a different one of the *meta* compounds. This study used much smaller doses (about 1.6 mg/kg) and fewer total dyeings (weekly for 18 months), the animals were sacrificed immediately after the last application, and as a whole the study is even more inadequate for the evaluation of safety for humans at an acceptable risk factor.

Carcinogenicity Studies on Other Hair Dye Chemicals. A N.C.I. study on feeding rodents 2-nitro-*p*-phenylenediamine and 4-nitro-*o*-phenylenediamine also has not been completed (U. Saffiotti, personal communication). The *para* compounds *p*-phenylenediamine and 2,4-diaminotoluene have been tested in the topical studies mentioned. The Cosmetic, Toiletry, and Fragrance Association has started a cancer and reproduction study, to test compounds in hair dyes by topical application in rodents.

In conclusion, we do not find that the reported cancer studies provide convincing evidence of hair dye safety in view of the variety of mutagenic compounds present in hair dyes, the carcinogenicity of many aromatic amines, and the close structural relationship of the mutagen 2,4-diaminoanisole to the carcinogen 2,4-diaminotoluene. If topical studies are to be among those used it is apparent that thousands of animals, higher doses, and lifetime studies are necessary to overcome the statistical limitations due to spontaneous tumors. In addition, it seems essential to use a relevant positive control (e.g., benzidine), to use rats and mice and perhaps other species (e.g., β-naphthylamine, a potent human carcinogen, is weak in rats and mice) (27), and to measure the actual absorption of the material in the test animal as compared to humans. It is particularly important to determine the kinds and amounts of chemicals actually excreted in human urine after hair dyeing (we are informed that industry now plans to do this). The *Salmonella*/microsome system could be used for detecting mutagenic metabolites in urine (7) of both rodents and people.

Benzidine Comparison. It is instructive to compare the amount of hair dye components absorbed in humans with the amount of a known human carcinogen which causes cancer. We estimate (see above) that the cumulative dose of hair dye chemicals absorbed in humans after 10 years of monthly hair dyeings may be between 10 mg/kg and 100

* A recent study (D. Hoel, personal communication) deals in detail with the problem of extrapolating data on carcinogenesis and mutagenesis from laboratory animals to man. They recommend that, for equivalent doses, a surface comparison be used (about the 2/3 power of the weight) (25, 26) rather than a straight mg/kg comparison. This would mean that the mg/kg dose for a human should be about 12-fold less relative to a mouse and 6-fold less relative to a rat for comparable results. In addition, any comparison has a high degree of uncertainty because of possible differential absorption, metabolism, and other factors.

mg/kg. In comparison, we discuss benzidine because its carcinogenicity has been studied in factory workers, it is an aromatic diamine, and it causes the same type of frameshift mutations (on strain TA1538) as the hair dye mutagens. The amount of benzidine absorbed per male factory worker has been estimated by M. Meselson (28) from the amount of benzidine found in urine, the recovery factor, and years of exposure, and this can be related to the known incidence of bladder cancer in the workers (29–31). An estimated cumulative dose per worker of 200 mg/kg gave 52% tumors among a group of American workers and an estimated 50 mg/kg cumulative dose gave 22% tumors among Italian workers. Although these estimates are not precise they give a feeling for the amounts involved. In a study (32) of the effect of benzidine on a strain of female rats sensitive to the induction of mammary tumors, a cumulative dose of about 40 mg/kg (12 mg per rat) gave 50% mammary tumors.

Epidemiology. About 30% of women in the U.S. dye their hair, often monthly and for many years. The possible risk of cancer and of genetic abnormalities appears sufficiently large that large scale epidemiological studies should be done to determine any relationship between cancer incidence and years of hair dyeing, or of industrial exposure in workers. Since mammary and bladder tumors can be caused by aromatic amines, a study of these tumor categories should certainly be included (though a carcinogen can often cause a different type of tumor in humans than in rodents). For example, breast cancer is the leading cancer in women in the U.S., its incidence is rising in younger and middle aged women (33, 34), is five times as high in the U.S. as in Japan, and is thought by many epidemiologists to have an environmental cause (35). It would also be of interest to look at the incidence of birth defects among children born to women who dye their hair: if these chemicals are mutagens for humans, the cost to society of damage to the germ line may be even greater than that due to cancer (18).

If hair dyeing chemicals are environmental carcinogens (or mutagens) for humans, we probably have only started to see the consequences. In fact, there is a very long delay period for the appearance of human cancer after the time of exposure (1–3 decades) and also an additional delay before sufficient numbers of cases are recorded, analyzed, and published. Moreover, hair dye use has not been constant, but has increased dramatically (perhaps 30-fold) over the last 30 years. Thus, current epidemiological studies might only recognize a tiny fraction of any cancers caused by hair dyeing in the past. Any past contributions of just 2,4-diaminotoluene and vinyl chloride (a propellent in cosmetic spray cans) undoubtedly have not yet made their full impact, and in any case it may be difficult to untangle these from potential contributions of the other chemicals in hair dyes.

The cosmetic industry is now testing hair dye components for carcinogenicity, even though they are not required to do so (10, 36). Our findings emphasize that any reevaluation of industry regulation should include the design of a system for testing environmental chemicals for carcinogenicity and mutagenicity so as to insure acceptable risk factors and objectivity.

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1. Ames, B. N. (1971) in *Chemical Mutagens*, ed. Hollaender, A. (Plenum Press, New York), Vol. 1, chap. 9, pp. 267–282.
2. Ames, B. N., Lee, F. D. & Durston, W. E. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 782–786.
3. Ames, B. N., Durston, W. E., Yamasaki, E. & Lee, F. D. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2281–2285.
4. Ames, B. N., Sims, P. & Grover, P. L. (1972) *Science* **176**, 47–49.
5. Ames, B. N., Gurney, E. G., Miller, J. A. & Bartsch, H. (1972) *Proc. Nat. Acad. Sci. USA* **70**, 3128–3132.
6. Isono, K. & Yourno, J. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 1612–1617.
7. Durston, W. E. & Ames, B. N. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 737–741.
8. Kier, L. D., Yamasaki, E. & Ames, B. N. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 4159–4163.
9. McCann, J., Spingarn, N., Kobori, J. & Ames, B. N. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 978–983.
10. Heenan, J. (1974) *FDA Consum.*, November, pp. 22–27.
11. Burnett, C., Lanman, B. Giovacchini, R., Wolcott, G., Scala, R. & Keplinger, M. (1975) *Food Cosmet. Toxicol.*, in press.
12. Corbett, J. F. (1971) in *The Chemistry of Synthetic Dyes* (Academic Press, New York), Vol. 5, pp. 475–534.
13. Corbett, J. F. (1973) *J. Soc. Cosmet. Chem.* **24**, 103–134.
14. Ito, N., Hiasa, Y., Konishi, Y. & Marugami, M. (1969) *Cancer Res.* **29**, 1137–1142.
15. Wernick, T., Lanman, B. M. & Fraux, T. L. (1975) *Toxicol. Appl. Pharmacol.*, in press.
16. Ritter, J. J. & Schmitz, G. H. (1929) *J. Amer. Chem. Soc.* **51**, 1587–1589.
17. Brody, F. & Burns, M. S. (1968) *J. Soc. Cosmet. Chem.* **19**, 361–379.
18. Report of the Environmental Mutagen Society (1975) *Science* **187**, 503–514.
19. Kiese, M. & Rauscher, E. (1968) *Toxicol. Appl. Pharmacol.* **13**, 325–331.
20. Kiese, M., Rachor, M. & Rauscher, E. (1968) *Toxicol. Appl. Pharmacol.* **12**, 495–507.
21. Meigs, J. W., Sciarini, L. J. & Van Sandt, W. A. (1954) *Arch. Ind. Hyg. Occup. Med.* **9**, 122–132.
22. Feldmann, R. J. & Maibach, H. I. (1970) *J. Invest. Dermatol.* **54**, 399–404.
23. Umeda, M. (1955) *Gann* **46**, 597–606.
24. Kinkel, H. J. & Holzmann, S. (1973) *Food Cosmet. Toxicol.* **11**, 641–648.
25. Rall, D. P. (1969) *Environ. Res.* **2**, 360–367.
26. Freireich, E. J., Gehan, E. J., Rall, D. P., Schmidt, L. M. & Skipper, H. E. (1966) *Cancer Chemother. Rep.* **50**, 219–244.
27. IARC Monograph on the Evaluation of Carcinogenic Risk (1974) (IARC, Lyon, France) **4**, 97–111.
28. *Study on Problems of Pest Control* (1975) National Academy of Sciences, in press.
29. Vigliani, E. C. & Barsotti, M. (1961) *Med. Lavoro* **52**, 241–250.
30. Zavon, M. R., Hoegg, U. & Bingham, E. (1973) *Arch. Environ. Health* **27**, 1–7.
31. Rinde, E. & Troll, W. (1975) *J. Nat. Cancer Inst.*, in press.
32. Griswold, D. P., Casey, A. E., Weisburger, E. K. & Weisburger, J. H. (1968) *Cancer Res.* **28**, 924–933.
33. Feinleib, M. & Garrison, R. J. (1969) *Cancer* **24**, 1109–1116.
34. Cutler, S. J., Christine, B. & Barclay, T. H. C. (1971) *Cancer* **28**, 1376–1380.
35. MacMahon, B., Cole, P. & Brown, J. (1973) *J. Nat. Cancer Inst.* **50**, 21.
36. Nader, R. (1974) in *Consumer Health and Product Hazards/ Cosmetics and Drugs, Pesticides, Food Additives*, eds. Epstein, S. S. & Grundy, R. D. (MIT Press, Cambridge, Mass.), chap. 2, pp. 73–141.

EXHIBIT D

SCCS/1479/12
Revision of 18 September 2012

Health and Consumers
Scientific Committees

Scientific Committee on Consumer Safety

SCCS

OPINION ON

Toluene-2,5-diamine and its sulfate

COLIPA n° A5

The SCCS adopted this opinion at its 15th plenary meeting

of 26 – 27 June 2012

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.

They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

Scientific Committee members

Jürgen Angerer, Ulrike Bernauer, Claire Chambers, Qasim Chaudhry, Gisela Degen, Elsa Nielsen, Thomas Platzek, Suresh Chandra Rastogi, Vera Rogiers, Christophe Rousselle, Tore Sanner, Jan van Benthem, Jacqueline van Engelen, Maria Pilar Vinardell, Rosemary Waring, Ian R. White

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http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

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This opinion has been subject to a commenting period of four weeks after its initial publication. Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged.
Revised opinions carry the date of revision.

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1. BACKGROUND

Submission I on toluene-2,5-diamine was submitted in December 1979 by COLIPA[1] according to COLIPA.

Submission II on toluene-2,5-diamine was submitted in July 2005 by COLIPA.

Submission III on toluene-2,5-diamine and its sulfate salt was submitted in October 2010 by COLIPA.

The Scientific Committee on Consumer Safety (SCCS) adopted at its 10th plenary meeting the 22 of March 2011 the opinion (SCCS/1390/10) with the following conclusion:

Based on the low Margin of Safety using the conventional risk assessment approach, toluene-2,5-diamine and its sulphate salt cannot be considered safe for use as an oxidative hair dye with a concentration on-head of maximum 4.0% (calculated as free base) or 7.2% (calculated as sulfate salt).
The kinetics-based approach for MoS calculation proposed by the applicant, using the AUC determined in a human *in vivo* exposure study, was not accepted due to the shortcomings of the underlying study which was not performed according to modern standards. In order to come to a final conclusion, the SCCS is of the opinion that a state of the art human exposure study *in vivo* would be required.
Toluene-2,5-diamine is at least a strong skin sensitiser.

The substance and its salts is currently regulated in entry 9a of Annex III to the Cosmetics Directive on the list of substances, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission IV on toluene-2,5-diamine was submitted by COLIPA in February 2012.

This submission contains the results and conclusions obtained in the new performed study in which the applicant assesses the safety of toluene-2,5-diamine at on head concentration of up to 1.5% (expressed as free base) in oxidative hair colouring products and conclude that concentration up to 2.0% toluene-2,5-diamine (expressed as free base) would also meet the requirements for a sufficient Margin of safety.

2. TERMS OF REFERENCE

1. *Does the SCCS consider toluene-2,5-diamine and its sulfate salt, safe for use as an oxidative hair dye with a concentration on-head of maximum 2.0% (3.6% calculated as sulfate salt) taken into account the scientific data provided?*

2. *If not, does the SCCS recommend any other concentration limit with regard to the use of toluene-2,5-diamine and its sulfate salt as an oxidative hair dye?*

3. *And/or does the SCCS recommend any further restrictions with regard to the use of toluene-2,5-diamine and its sulfate salt, in oxidative hair dye formulations?*

[1] COLIPA – The European Cosmetics Association

3. OPINION

3.1. Chemical and Physical Specifications

Toluene-2,5-diamine is used in hair dyes in the form of its free base or its sulfate salt.

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

Toluene-2,5-diamine (INCI)
Toluene-2,5-diamine sulfate (INCI)

3.1.1.2. Chemical names

Free Base
1,4-Benzenediamine, 2-methyl- (CA INDEX NAME, 9CI)

Sulfate
2,5-Diaminotoluene sulfate
2-Methyl-p-phenylenediamine sulfate
Toluenediamine sulfate
p-Toluenediamine sulfate
p-toluylenediamine sulfate

3.1.1.3. Trade names and abbreviations

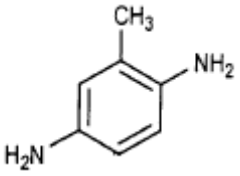
Free base
Imexine OD
COLIPA A005
Colour Index no 76042

Sulfate
Colorex 25DTS (Chemical Compounds, Inc.)
Jarocol TDS (Robinson)
Rodol BLFX (Lowenstein)
COLIPA no A005
Colour Index no 76043

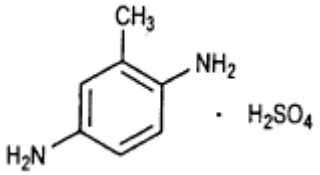
3.1.1.4. CAS / EC number

	Free Base	*Sulfate*
CAS:	95-70-5	615-50-9 (sulfate 1:1); 6369-59-1 (sulfate 1:x)
EC:	202-442-1	210-431-8 (sulfate 1:1); 228-871-4 (sulfate 1:x)

3.1.1.5. Structural formula



Free base



Sulfate

3.1.1.6. Empirical formula

	Free base	Sulfate
Formula:	$C_7H_{10}N_2$	$C_7H_{10}N_2.H_2O_4S$

3.1.2. Physical form

Free base: light yellow to light pink
Sulfate: grey to white powder

3.1.3. Molecular weight

	Free base	*Sulfate*
Molecular weight:	122.17	220.25

3.1.4. Purity, composition and substance codes

Batches used (survey on all the files of Submission II)

Toluene-2,5-diamine (50% aqueous solution)

This name and the respective data below were found only in the "A5 SUMMARY submission II 2005.doc" (pages 8-10). Instead of batch numbers, in page 9 it is noted "*See Annex I for summary and Reference: 3"*. However, Annex 1 and ref. 3 refer only to the sulfate salt.

Toluene-2,5-diamine sulfate

- Batch 2346/01-R99053665 (abbreviations: Batch 2346 or Batch R99053665)
- Batch EFH 290394
- Batch CH1143
- Batch 46847
- Lot 16825DR Sigma Aldrich (see Study 2, human hepatic metabolism *in vitro)*
- Batch präp. 139 (Purity: 98.2%; see 3.3.8.1. Two generation reproduction toxicity)
- Batch 23005

A complete characterization is provided for the first two batches only.

Radioactive toluene-2,5-diamine sulfate

- 3362-259 [ring-U-^{14}C]-toluene-2,5-diamine sulfate (radiochemical purity 99.3% by HPLC)
- CFQ13783, batch 1 [ring-U-^{14}C]-toluene-2,5-diamine sulfate (radiochemical purity 98.2% by HPLC)

Purity, accompanying contaminants, and batch codes

Toluene-2,5-diamine (50% aqueous solution)	Toluene-2,5-diamine Sulfate
Purity HPLC relative > 99% Potentiometric Titer: 48-52% Potential impurities o-Toluidine * < 20 ppm	Purity HPLC quantitative > 96.3 weight% NMR quantitative > 97.3 weight% Potential impurities o-Toluidine < 8 ppm
Solvent residues Solvents (i.e. solvents such as methanol, ethanol, isopropanol, n-propanol, acetone, ethyl acetate, cyclohexane, methyl ethyl ketone and monochlorobenzene < 100 ppm) were not detected.	

* EU CMR classification: carcinogen category 1B (regulation (EC) No. 1272/2008)

Ref.: 3

Material used in the market (Deduced specifications)

Toluene-2,5-diamine (50% aqueous solution)	Toluene-2,5-diamine sulfate
Purity HPLC qualitative: > 99% Potentiometric Titer: 48-52% Potential impurities o-Toluidine < 50 ppm	Purity HPLC quantitative: > 98% w/w HPLC qualitative (254 nm) > 99% Solvent content: < 1% Potential impurities o-Toluidine < 50 ppm

Comparison of two different batches of toluene-2,5-diamine sulfate

	batch EFH 290394 29.03.1994	batch 2346 (/01, R99053665) 09.12.1999
NMR content / weight%	97.3	99.5
HPLC purity / area% 210 nm 254 nm 303 nm	 99.3 99.0 99.6	 99.5 99.7 (at 290 nm)
HPLC quantitative (compared to R99053665)	101.6%	
Loss on drying / weight%	*	0.20
Water content / weight%	*	0.04
Sulfated ash / weight%	*	0.11
o-Toluidine	< 1 ppm (detection limit)	< 1 ppm (detection limit)
UV spectra (ethanol)		
ε_{mol} (242 nm) / l cm^{-1} mol^{-1}	9820	*
ε_{mol} (308 nm) / l cm^{-1} mol^{-1}	2419	*

* not determined

3.1.5. Impurities / accompanying contaminants

See point 3.1.4.

3.1.6. Solubility

Solubility of toluene-2,5-diamine

A005 50% aqueous solution:

in water	≥ 10 g/100ml
in ethanol 100%	≥ 10 g/100ml
in DMSO	≥ 10 g/100ml

Ref.: 115

Solubility of toluene-2,5-diamine has not been determined according to standard methods (e.g. EU - A.6)

Solubility of toluene-2,5-diamine sulfate

in water:	5.03 g/l (20 °C) (EU - A.6)	Ref.: 6
in acetone / water 1:1:	< 1 g/l	
in DMSO:	5 < S < 15 g/l	
in ethanol:	1 < S < 10 g/l	(taken from submission summary)

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}:	0.74 (sulfate)	(HPLC method, EU method A.8)
	- 0.32 (free base, 50% aqueous solution)	(shake-flask method, EU method A8)
	0.160 (free base)	(ref. 115)

3.1.8. Additional physical and chemical specifications

Physical properties of toluene-2,5-diamine sulfate

Appearance:	grey to white powder		
Particle size distribution:	mean particle diameter: 46 µm (CIPAC MT59)		Ref. 4
Particle size distribution:	median particle size L50 = 6.5 µm (by laser diffraction ; OECD 110,CIPAC MT 187)		Ref. 5
pH-value:	2.47 (20 °C ; saturated aqueous solution)		Ref. 6
pKa-value:	6.39 and 2.77 (calculated, Pallas Software)		Ref. 7
Melting point:	not detectable, decomposed at 240 °C	(EU – A.1)	Ref. 8
Boiling point:	not detectable, decomposed at 240 °C	(EU – A.2)	Ref. 8
Density:	1.366 g/ml (20 °C)	(EU - A.3)	Ref. 9
Vapour pressure:	<1.0 exp - 7 hPa (20 °C)	(EU - A.4)	Ref. 10
Flammability (solids):	not highly flammable	(EU - A.10)	Ref. 12
Relative self-ignition Temperature:	327 °C	(EU - A.16)	Ref. 13
Surface tension (in water):	69.7 mN/m (20 °C)	(EU - A.5)	Ref. 11
Refractive index:	/		
UV_Vis spectrum	λ_{max} = 210 nm, 254 nm, 303 nm		

Physical properties of toluene-2,5-diamine (free base)

Melting point:	64 °C
Boiling point:	273.5 °C
Vapour pressure:	3.40E-03 (0.0034) mm Hg at 25 °C

Henry's Law Constant: 7.43E-09 (7.43 10^{-9}) atm-m³/mole at 25 °C

Ref.: 115

3.1.9. Stability

The stability of the test substance in aqueous and water:acetone (4:1, v/v) solution was monitored over a time period of 8 days. During the test procedure, the aqueous and water:acetone stock solutions were stored at ambient temperature in the absence of light. The recoveries of the test substance in both solvents were 99.7–109%.

General Comments on physico-chemical characterisation

* Batch 46847, used in 3 mutagenicity studies (ref. 38, 40 and 41), batch 23005, used in the teratogenicity studies (ref. 53 and 54) as well as batches CH1143, präp. 139 and Lot 16825DR Sigma Aldrich were not characterised
* The stability of toluene-2,5-diamine and its sulfate in typical hair dye formulations was not reported.
* The impurity o-toluidine is classified by the EU as carcinogenic category 1B (regulation (EC) No. 1272/2008).
* No documentation was provided to support the reported data on the free base.
* Solubility of toluene-2,5-diamine has not been determined according to standard methods (e.g. EU - A.6)

3.2. Function and uses

Toluene-2,5-diamine and its sulfate are used as an oxidative hair colouring agent (precursor). The intended maximum on-head concentration is 2.0% (calculated as free base), or 3.6% (calculated as sulfate).

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline: /
Species/strain: rat, CFY strain
Group size: 5 males and 5 females per dose group
Test substance: toluene-2,5-diamine
Batch: /
Purity: /
Dose: 0, 64, 100, 160 and 250 mg/kg bw
Route: oral, gavage
Exposure: once
GLP: /

The test substance was diluted at 10% in aqueous sodium sulphite (0.05%) and administered once by oral gavage. During the observation period of 14 d mortalities and signs of toxicity were recorded and body weight was measured weekly.

Results
Lethargy, piloerection, ataxia and increased salivation were observed shortly after dosing. At 100 mg/kg bw increased respiratory rate and above 100 mg/kg bw decreased respiratory rate were observed. The acute median lethal oral dose and its 95% confidence limits were calculated to be 102 (69 – 152) mg/kg bw.

Dosage (mg/kg bw)	**Mortality (number deaths/number dosed)**	
	Male	**Female**
0	0/5	0/5
64	3/5	0/5
100	4/5	3/5
160	0/5	5/5
250	5/5	4/5

Ref.: 16

Comment
Despite the lack of data on the batch used and although the study does not conform to OECD guidelines, it is useful for evaluation.

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline: OECD 404 (1992)
Species/strain: Rabbit / New Zealand White
Group size: 3 Males
Test substance: Imexine OD (free base)
Vehicle: None (test substance consisted of active ingredient with water)
Batch: op T 784
Purity: 50.6% active (in water)
GLP: In compliance

A single dose of 0.5 ml of the test substance (pH 9.71) was prepared on a dry compress and then applied to a 6 cm^2 clipped area of the skin and covered with a semi-occlusive dressing for 4 h. Skin reactions were evaluated 1 h, 24 h, 48 h, and 72 h after removing the dressing and then daily until day 6.

Results
No oedema, ulceration or necrosis was noted. Evaluation of erythema was not possible due to the black colouration of the treatment site.

Conclusion
Although the application of a 50.6% aqueous solution of Imexine OD produced no evidence of an oedematous response after topical application under semi-occluded conditions in New Zealand White rabbits, an erythematous response could not be excluded due to black colouration of the treatment site by the test substance.

Ref.: 17

Guideline: /
Species/strain: Rabbit / New Zealand White
Group size: 3 Males
Test substance: toluene-2,5-diamine
Vehicle: water
Batch: /
Purity: /
GLP /

0.5 ml of a 2.5% w/v solution of toluene-2,5-diamine in aqueous 0.05% sodium sulphite (pH 7.0) was tested on intact and abraded skin of three New Zealand White rabbits under occlusive patches. Cutaneous reactions were observed at 24 h (immediately after patch removal) and again at 72 h.

Results
Slight erythema with and without very slight oedema was observed in the intact and abraded sites, respectively, of one animal at the 24 h evaluation. At 72 h no signs of irritation were observed.

Conclusion
The test substance was considered to be mildly irritating to rabbit skin under the conditions of this test.

Ref.: 18

Comments
In an *in vivo* study in rabbits, a 50.6% Imexine OD applied under semi-occlusive conditions did not produce evidence of oedema and could not be evaluated for erythema due to black colouration of the skin. In the second experiment, which did not conform to guidelines or GLP, the test substance was irritant to rabbit skin under occlusive conditions.

3.3.2.2. Mucous membrane irritation

Guideline: OECD 405
Species/strain: Rabbit / New Zealand White
Group size: 1 Male
Test substance: Imexine OD (free base)
Vehicle: None (test substance consisted of active ingredient with water)
Batch: op T 784
Purity: 50.6% active (in water)
GLP: In compliance

A volume of 0.1 ml of the test substance (pH 9.71) was applied into the conjunctival sac of the left eye of one male rabbit; the right eye served as a control. The eye was not rinsed, and was evaluated and scored according to the Draize scoring system at 1, 24, 48, and 72 h after application and then daily until Day 8.

Results
The test substance induced marked conjunctival irritation with chemosis and redness, slight iridial irritation and moderate to slight corneal opacity. All of these effects were reversible within 7-8 days.

Conclusion
Under the test conditions, in which the test substance was applied undiluted and was not rinsed from the eye, the test substance was irritating to the rabbit eye. The high pH of the test solution may have contributed to the observed irritation.

Ref.: 19

Guideline: /
Species/strain: Rabbit / New Zealand White
Group size: 3 Males
Test substance: toluene-2,5-diamine
Vehicle: water
Batch: /
Purity: /
GLP: /

0.1 ml of a 2.5% w/v solution of toluene-2,5-diamine in 0.05% aqueous sodium sulphite (pH 7.0) was instilled into one eye of each of three rabbits. After 10 seconds the eye was rinsed with 50 ml of lukewarm water. Eyes were evaluated and scored according to the Draize scoring system at 1 h and then at Days 1, 2, 3, 4, and 7.

Results
Mild conjunctival irritation was observed in 2 animals on days 1 and 3 respectively.

Conclusion
Under the conditions of this test, a 2.5% toluene-2,5-diamine solution caused slight irritation to rabbit eyes.

Ref.: 20

Acute eye irritation potential in vitro: HET-CAM

Guideline: /
Species/strain: White Leghorn chicken eggs, freshly fertilized
Group size: 6 eggs
Test substance: p-toluenediamine sulfate (code SAT 010935)
Batch: 46847
Purity: 99.9%
GLP: /

The test substance was tested undiluted for its irritation potential on the chorioallantoic membrane of fertilized chicken eggs using the endpoint assessment for non-transparent solid test items.

Texapon ASV 70 (sodium magnesium laurylmyristyl-6-ethoxy-sulfate) at a test concentration of 5% was used as reference substance with this internal benchmark being defined to be moderately irritating to the rabbit eye *in vivo*.

The undiluted test substance was applied to the chorioallantoic membrane and then rinsed off with physiological saline after 30 sec. Endpoints (haemorrhage, coagulation, and blood vessel lysis) were assessed and semi-quantitatively scored at 30 sec (reference substance) or 180 sec (test substance) after rinsing.

Results
For the relevant endpoints of haemorrhage, coagulation and lysis, scores of 0, 0, and 0, respectively, were obtained with p-toluenediamine sulfate. With Texapon ASV 70 scores of 12, 9, and 0, respectively, were obtained.

Conclusion
Based on the results of this test, p-toluenediamine sulfate was predicted to be 'no more than slightly irritating to mucous membranes', including the eye. The results with the reference substance, Texapon ASV 70, were indicative of a moderately irritating effect.

Ref.: 21

Comment on status of HET-CAM
The HET-CAM (Hen's Egg Test-Chorio Allantoic Membrane) provides only supportive evidence for cosmetic ingredient safety assessment. This method can be recommended for use as screening tests for the identification of ocular corrosives and severe irritants, the protocol and decision criteria for the identification of ocular corrosives and severe irritants need to be optimized and undergo further validation (SCCS/1294/10).

Comment on eye irritation
Eye irritation studies have demonstrated that undiluted Imexine OD is irritant to the rabbit eye. Some irritant effects were also seen with 2.5% toluene-2,5-diamine solution in rabbits and undiluted toluene-2,5-diamine sulfate in the HET-CAM test. The intended on-head concentration is up to 3.6%.

3.3.3. Skin sensitisation

Animal data

Local Lymph Node Assay (LLNA)

Study 1

Reference 22 is the same study as ref. 24, below. Stimulation indices (SI) of 4.4, 10.4 and 19.4 were obtained from test concentrations of 0.5, 1.5 and 2.8%. An EC_3 value of 0.31% was derived by linear regression, indicating that the substance is a strong skin sensitiser in this experiment.

	Study reference 22	**Study reference 24**
Guideline:	/	OECD 406
Species:	mice, CBA/ca01aHsd	mice, CBA/ca01aHsd
Group:	5 animals per test group	female, 5 animals per test group, 3 test groups, 1 positive control group, 1 negative control group
Substance:	p-toluenediamine sulfate (PTD)	Haarbraun, 2-methyl-1,4-benzenediamine sulphate
Batch:	/	2346
Purity:	/	99.5 area% (254 nm) and 99.7 area% (290 nm)
Dose:	25 µl of PTD at 0.5, 1.5 and 2.8%	25 µl of the substance at 0.5, 1.5 and 2.8%
Vehicle:	Aqua/acetone/olive oil (AAOO) 2:2:1	Aqua/acetone/olive oil (AAOO) 2:2:1
Control:	/	p-phenylenediamine 1% in AAOO
GLP:	/	in compliance

In reference 24, the skin sensitising potential of the test substance was investigated by measuring the cell proliferation in the draining lymph nodes after topical application on the ear. 25 µl containing 0 (vehicle only), 0.5, 1.5 and 2.8% of the test substance in a mixture of aqua/acetone (1:1) with olive oil (4:1) were applied to the surface of the ear to each of five mice per group for three consecutive days. p-Phenylenediamine (PPD) at 1% in AAOO was used as the positive control in parallel under identical test conditions

On day 5, the mice received an intravenous injection of 250 µl phosphate buffered saline containing 20 µCi of [^{3}H]-methyl thymidine. Approximately five hours later, the mice were sacrificed by CO_2-inhalation and the draining auricular lymph nodes were removed. After preparing a single cell suspension for each mouse, cells were precipitated by TCA and the radioactivity was determined (incorporation of [^{3}H]-methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm). The mean dpm per treated group was determined and the stimulation index (test item compared to the concurrent vehicle control) was calculated.

Results
Mean stimulation indices (SI) of 4.4, 10.4 and 19.4 were obtained for the test concentrations of 0.5, 1.5 and 2.8%, respectively. No EC_3 value was calculated, since all stimulation indices were above 3. The positive control (PPD 1% in AAOO) caused a stimulation index of 5.3.

Ref.: 22, 24

Comment
The lowest concentration used in this test was too high. An extrapolated EC_3 value was calculated by linear regression in reference 22 (found to be 0.31%).
References 22 and 24 seem to use the same data and describe the same LLNA study, however with a different presentation.

Study 2

Guideline:	OECD 406
Species:	mice, CBA/ca01aHsd
Group:	female, 5 animals per test group, 3 test groups, 1 positive control group, 1 negative control group
Substance:	Haarbraun, 2-methyl-1,4-benzenediamine sulphate
Batch:	2346
Purity:	99.5 area% (254 nm) and 99.7 area% (290 nm)
Dose:	25 µl of the substance at 0.5, 1.5 and 5.0%
Vehicle:	DMSO
Control:	p-phenylenediamine 1% in DMSO
GLP:	in compliance

The skin sensitising potential of the test substance was investigated by measuring the cell proliferation in the draining lymph nodes after topical application on the ear. 25 µl containing 0 (vehicle only), 0.5, 1.5 and 5.0% of the test substance in DMSO were applied to the surface of the ear to each of five mice per group for three consecutive days. p-Phenylenediamine (PPD) at 1% in DMSO was used as the positive control in parallel under identical test conditions

On day 5, the mice received an intravenous injection of 250 µl phosphate buffered saline containing 20 µCi of [^{3}H]-methyl thymidine. Approximately five hours later, the mice were sacrificed by CO_2-inhalation and the draining auricular lymph nodes were removed. After preparing a single cell suspension for each mouse, cells were precipitated by TCA and the radioactivity was determined (incorporation of [^{3}H]-methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm). The mean dpm per treated group was determined and the stimulation index (test item compared to the concurrent vehicle control) was calculated.

Results
Mean stimulation indices (SI) of 4.9, 4.2, and 3.7 were obtained for the test concentrations of 0.5, 1.5 and 5%, respectively. No EC_3 value was calculated, since all stimulation indices were above 3. The positive control (PPD 1% in DMSO) caused a stimulation index of 10.1

Ref.: 25

Comment
The lowest concentration used in this test was too high and therefore the study is inadequate.

Guinea pig studies

Guideline:	/
Species/strain:	Female Hartley strain albino guinea pigs
Group size:	6 animals in each test group and each control group The number of control groups was not given
Test substances:	p-toluenediamine 2HCl (PTD); p-phenylenediamine (PPD) p-aminophenol (PAP); p-aminoazobenzene (PAB); Sudan III
Batch:	/
Purity:	PTD: 98%; PPD: 97%; PAP: 98%; PAB: 98%; Sudan III: 99%
Concentrations:	*Intradermal induction*: 0.1% test substance in saline (PTD and PPD) or in olive oil (PAP, PAB, and Sudan III), and in Freund's complete adjuvant (FCA)/saline *Topical induction*: 1% test substance in petrolatum, occluded Pre-treatment with 10% sodium lauryl sulfate in petrolatum

Challenge: 0.001, 0.01 and 0.1% test substance in acetone or in acetone/distilled water, open application

GLP: /

The aim of the study was to evaluate the skin sensitising potency of PTD, PPD, PAP, PAB and Sudan III, and to study cross-reactivity. Induction was performed according to the guinea pig maximisation test protocol by injections on day 0, and topical application on day 7 for 48 hours. Modifications included that the highest possible elicitation concentrations were not chosen, and that challenge was performed by open application and not closed. Challenge on day 21 by open application for 24 hours. Readings were made at 24, 48 and 72 hours after challenge application.

Results

Only results related to PTD are reviewed here. 100% of the animals induced with PTD (6/6) reacted at challenge with PTD, showing that the test substance was an extremely potent skin sensitizer (Table 1). Positive reactions were recorded in the animals induced with PTD at challenge also with PPD (5/6), PAP (3/6), PAB (5/6) and Sudan III (1/6), indicating cross-reactivity to these substances in animals induced with PTD (Table 1). 100% of the animals induced with PPD (6/6) reacted at challenge with PTD, but none of the animals induced with PAP or PAB (Table 2). The results indicate cross-reactivity to PTD in animals induced with PPD.

Table 1: Sensitisation and cross-reactivity test in guinea pigs induced with PTD. Response at challenge with PTD, PPD, PAP, PAB or Sudan III

	Challenge substance (no. positive at challenge/no. induced)				
Challenge concentration (%)	**PTD**	**PPD**	**PAP**	**PAB**	**Sudan III**
0.1	6/6	5/6	3/6	5/6	1/6
0.01	5/6	2/6	0/6	5/6	1/6
0.001	0/6	0/6	0/6	1/6	0/6

Table 2: Sensitisation and cross-reactivity test in guinea pigs induced with PPD, PAP, PAB or Sudan III. Response at challenge with PTD

	Induction substance (no. positive at challenge/no. induced)		
PTD challenge concentration (%)	**PPD**	**PAP**	**PAB**
0.1	6/6	0/5	0/6
0.01	0/6	0/5	
0.001	0/6		

Conclusions

Although not performed according to guideline, the results indicate that PTD is an extreme skin sensitiser. The results indicate also that cross-reactivity in animals induced with PTD occurs to PPD and PAB; and to PTD in animals induced with PPD. As contaminants in test substances were not analysed, conclusions concerning cross-reactivity remain limited.

Ref.: 26

Guideline: /
Species/strain: Hartley albino guinea pigs
Group size: 10 animals in each pre-test group
The number of animals in test groups was not given, no control group
Test substances: toluene-2,5-diamine sulfate
Batch: /

Purity: /
Concentrations: *Topical induction*: 1% in petrolatum, occluded
Challenge: 0.01, 0.05, 0.1, 0.2, 0.5 and 1% test substance, occluded
GLP: /

The aim was to assess the skin sensitising potency in the guinea pig of ten dye intermediates, including toluene-2,5-diamine sulphate, and to compare the results with results from patch testing hair colouring dermatitis patients in Japan. The study was performed by a non-guideline method. Pre-tests were performed by occluded exposure to determine the irritancy threshold. Topical induction was performed by occluded exposure for 48 hours on the nape, 3 times per week for two weeks. Following a 2 week rest period, challenge was performed by occluded exposure for 48 hours on the flank. Readings were made at 24 and 48 hours after removal of the test material. It was reported that 40% of the animals reacted positively to toluene-2,5-diamine sulfate at challenge with 1%, and 10% at challenge with 0.10%.

Ref.: 27

Comment
The results of the study are of limited use.

Human data
Diagnostic patch testing

66 dermatitis patients (hairdressers) were patch tested with the North American patch test standard tray and a hairdresser series. 7.5% were positive to toluene-2,5-diamine sulfate, 46% were positive to p-phenylenediamine, 5% to p-aminodiphenylamine, 3% to o-nitro-p-phenylenediamine. (Table 1)

Ref.: 63

597 dermatitis patients (hairdressers), of which 61.8% were current hairdressers, were patch tested in an IVDK multi-centre study in Germany with the patch test standard series and the hairdressers' series. 21.4% were positive to toluene-2,5-diamine, 18.1% were positive to p-phenylenediamine, 4.0% to p-aminophenol, 3.4% to m-aminophenol. Results from previous periods were also presented - 14.3% were tested positive to toluene-2,5-diamine in 1990-1991 and 16.2% in 1993-1995. (Table 1)

Ref.: 64

106 dermatitis patients (hairdressers) in Greece (102 females and 4 males) were patch tested with the patch test standard series and the hairdressers' series. 10.3% were positive to toluene-2,5-diamine sulfate, 30.2% were positive to p-phenylenediamine, 8.4% to o-nitro-p-phenylenediamine, 4.7% to resorcinol, 4.3% to p-aminodiphenylamine, 2.8% to p-aminophenol. (Table 1)

Ref.: 65

In a multi-centre study by the European Environmental and Contact Dermatitis Research Group (EECDRG), a total of 809 dermatitis patients (hairdressers) were patch tested with hairdresser allergens in 9 centres. 7.6% were positive to toluene-2,5-diamine sulfate, 14.8% to p-phenylenediamine, 4.1% to o-nitro-p-phenylenediamine, 0.6% to resorcinol and 3.6% to p-aminodiphenylamine hydrochloride (Table 1).
In the same study, a total of 104 dermatitis patients identified as hairdressers' clients were patch tested with hairdresser allergens in 4 centres. 8.7% were positive to toluene-2,5-diamine sulfate, 19.2% to p-phenylenediamine, 7.7% to o-nitro-p-phenylenediamine, 1.9% to resorcinol and 3.9% to p-aminodiphenylamine hydrochloride (Table 4).

Ref.: 67

In a multi-centre study by the Italian Contact Dermatitis Research Group (GIRDCA), a total of 302 dermatitis patients (hairdressers) (259 females and 43 males) were patch tested with hairdressers' allergens in 9 Italian centres. 13.2% were positive to toluene-2,5-diamine sulfate, 16.6% to p-phenylenediamine base (in 1989-1990), 7.6% to p-phenylenediamine dihydrochloride (in 1985-1988), 7.9% to o-nitro-p-phenylenediamine, 1.3% to resorcinol and 10.6% to p-aminodiphenylamine. (Table 1)

Ref.: 68

In a multi-centre study by the German Contact Dermatitis Group (DKG), 178 dermatitis patients (hairdressers) were patch tested with hairdressers' allergens in 11 centres. 18.0% were test positive to toluene-2,5-diamine, 8.4% to toluene-2,5-diamine sulfate, 18.0% to p-phenylenediamine base, 0.6% to resorcinol, 1.1% to 3-aminophenol, 2.2% to p-aminodiphenylamine hydrochloride, 3.4% to 4-aminophenol and 6.2% to o-nitro-p-phenylenediamine. (Table 1)

Ref.: 69

103 hairdressers (not dermatitis patients) in the Netherlands (96 females and 8 males) were patch tested with a special series including standard allergens and hairdressers' allergens. 2% were positive to toluene-2,5-diamine sulfate, 6% to p-phenylenediamine and 4% to 2-nitro-4-phenylenediamine. (Table 1)

Ref.: 66

The degree and pattern of hand eczema in hairdresser trainees and hairdressers was compared in Norway. 75 hairdressers affected by hand eczema and 74 hairdresser trainees with or without hand eczema were examined and patch tested with a hairdressers' series and some additional substances from the standard series. 2.7% of the hairdressers affected by hand eczema were test positive to toluene-2,5-diamine sulfate, compared to 0% of the hairdresser trainees. (Table 2)

Ref.: 71

In a German multi-centre study by the IVDK, hairdressing cosmetics and hair care products were considered causative of contact dermatitis in a total of 2328 dermatitis patients (92% female). 884 of the cases were currently or had been working as hairdressers. 1217 had not been hairdressers (in the publication called clients). All were patch tested in 1995-2002. Among the hairdressers, 24.8% were test positive to toluene-2,5-diamine, 22.0% to p-phenylenediamine, 6.1% to p-aminophenol and 3.6% to m-aminophenol (Table 1). Among the non-hairdressers, 13.2% were test positive to toluene-2,5-diamine, 14.7% to p-phenylenediamine, 6.5% to p-aminophenol and 4.2% to m-aminophenol (Table 4)

Ref.: 72

209 dermatitis patients (hairdressers) in Italy (182 females and 27 males) were patch tested with a standard series and a hairdressers' series. 13.8% were positive to toluene-2,5-diamine sulfate, 36.8% to p-phenylenediamine base, 3.8% to p-aminodiphenylamine, 4.7% to o-nitro-p-phenylenediamine and 0.9% to resorcinol. (Table 1)

Ref.: 73

1000 dermatitis patients in Germany were patch tested with a standard series in 1970-1972 and another 1000 dermatitis patients were tested in 1976-1979. In 1970-1971, 2.3% were test positive to toluene-2,5-diamine (female 1.9%, male 2.9%), and 5.2% to Ursol (=p-phenylenediamine) (female 4.2%, male 6.6%). In 1997-1979, 3.4% were test positive to toluene-2,5-diamine (female 2.1, male 5.0%) and 7.0% to p-phenylenediamine (female 7.1%, male 6.8%). (Table 3)

Ref.: 74

5348 dermatitis patients were patch tested with a standard series in Hamburg, Germany. 2.7% were test positive to toluene-2,5-diamine (females 1.9%, males 3.8%) and 4.1% were test positive to p-phenylenediamine (females 4.2%, males 3.9%). (Table 3)

Ref.: 75

5202 dermatitis patients were patch tested in Belgium with a standard series and many patients were tested also with supplementary substances. 1.6% were test positive to toluene-2,5-diamine, 7.2% to p-phenylenediamine, 0.2% to resorcinol, 1.8% to o-nitro-PPD, 2.1% to p-aminodiphenylamine, 0.1% to p-toluene sulfate. (Table 3)

Ref.: 76

1385 dermatitis patients (824 females, 561 males) were patch tested in Vienna, Austria with a standard series. 2.5% were test positive to toluene-2,5-diamine, 3% to p-phenylenediamine and 0.4% to resorcinol. (Table 3)

Ref.: 77

261 dermatitis patients identified as hairdressers' clients, for whom treatment with hair dyes or permanent wave solutions was suspected to be the cause of the dermatitis (256 females, 5 males), were patch tested in Bologna, Italy with the Italian standards series for patch testing and with a hairdressers' screening series. 4.6% were test positive to toluene-2,5-diamine sulfate, 7.3% to p-phenylenediamine, 4.2% p-aminodiphenylamine, 4.6% to o-nitro-p-phenylenediamine and 0.4% to resorcinol. (Table 4)

Ref.: 78

154 dermatitis patients with a positive patch test reaction to p-phenylenediamine were tested further with para compounds frequently used in hair dyes, in Amsterdam, the Netherlands. 9.7% were positive to toluene-2,5-diamine sulfate, 15% to p-aminoazobenzene, 3.2% to p-aminophenol, 3.2% to o-nitro-p-phenylenediamine, 2.6% to p-aminodiphenylamine and 0.6% to resorcinol. (Table 4)

Ref.: 79

475 dermatitis patients for whom contact allergy to cosmetic ingredients had been shown by patch testing in 5 European centres in the UK, Germany and Belgium, were included in a retrospective study. 11 cases (possibly 2.3%) were tested positive to toluene-2,5-diamine, 33 cases to p-phenylenediamine, 8 cases to 2-nitro-p-phenylenediamine, 2 cases to n-phenyl-p-phenylenediamine, 1 case to resorcinol. It was not stated if all patients had been tested with all substances. (Table 4)

Ref.: 80

613 dermatitis patients had been patch tested with the German Contact Dermatitis Group (DKG) para-amino compounds test series. 10.0% were tested positive to toluene-2,5-diamine, 14.1% to p-phenylenediamine, 3.1% to p-aminophenol and 16.2% to p-aminoazobenzene. (Table 4)

Ref.: 81

819 dermatitis patients (589 females, 230 males, 1-93 years) in Belgium were patch tested with the standard series and from 16 years of age also with a complementary cosmetic-medicinal series, and depending on clinical history with additional tests. 0.6% were test positive to toluene-2,5-diamine, 2% to p-phenylenediamine, 0.2% to 3-aminophenol, 2-nitro-phenylenediamine and to 4-aminophenol. (Table 3)

Ref.: 82

Table 1: Contact allergy to toluene-2,5-diamine in patch tested dermatitis patients who were, or had been hairdresser. Test substance: toluene-2,5-diamine (TDA) or toluene-2,5-diamine sulphate (TDAs) 1% in petrolatum

Test substance	No. tested patients	Positive patch test (%)	Year	Country	Ref.
TDAs	66	7.6	1973-1981	Canada	63 Lynde
TDA	597	21.4	1996-1998	Germany	64 Uter
TDAs	106	10.3	1985-1994	Greece	65 Katsarou

TDAs	781	7.6	1988-1991	9 European centres	67 EECDRG
TDAs	302	13.2	1985-1990	Italy	68 Guerra
TDA TDAs	178	18.0 8.4	1988-1989	Germany	69 Frosch
TDA	884 a)	24.8	1995-2002	Germany	72 Uter
TDAs	209	13.8	2002	Italy	73 Iorizzo
SUMMARY	**3123**	**Mean: 16.8%**			

a) hairdresser dermatitis patients with dermatitis from hair cosmetics

Table 2: Contact allergy to toluene-2,5-diamine in patch tested hairdressers and hairdresser trainees. Test substance: toluene-2,5-diamine sulphate (TDAs) 1% in petrolatum

Test substance	Population	No. tested	Positive patch test (%)	Year	Country	Ref.
TDAs	Hairdressers in saloons	103	2%	1989-1992	The Netherlands	66 van der Walle
TDAs	Hairdressers with hand eczema	75	2.7%	1994	Norway	71 Holm
TDAs	Hair-dresser trainees, with or without hand eczema	74	0%	1994	Norway	71 Holm

Table 3: Contact allergy to toluene-2,5-diamine in patch tested unselected dermatitis patients. Test substance: toluene-2,5-diamine (TDA) or toluene-2,5-diamine sulphate (TDAs) 1% in petrolatum

Test substance	No. tested patients	Positive patch test (%)	Country	Year	Ref.
TDA	1000 1000	2.3 3.4	Germany	1970-1971 1976-1979	74 Schwarz
TDA a)	5348	2.7	Germany	1976-1980	75 Kuhlwein
TDA	5202	1.6	Belgium	Not specified	76 Broueckx
TDA	1386	2.5	Austria	1972-1976	77 Jarisch
TDA	819	0.6	Belgium	1998-1999	82 Kohl
SUMMARY	**14755**	**Mean: 2.5%**			

a) 0.25% pet.

Table 4: Contact allergy to toluene-2,5-diamine in patch tested dermatitis patients selected due to symptoms or exposure related to cosmetics. Test substance: toluene-2,5-diamine (TDA) or toluene-2,5-diamine sulphate (TDAs) 1% in petrolatum

Test substance	No. tested patients and selection criteria	Positive patch test (%)	Country	Year	Ref.
TDAs	104 Hairdressers' clients	8.7	4 European centres	1988-1991	67 EECDRG
TDA	1217 Dermatitis from hair cosmetics, not hairdressers	13.2	Germany	1995-2002	72 Uter
TDAs	261 Hairdressers' clients	4.6	Italy	1985-1990	78 Guerra
TDAs	154 Patch-test pos. to PPD	9.7	The Netherlands	1996-1999	79 Koopmans

Test substance	No. tested patients and selection criteria	Positive patch test (%)	Country	Year	Ref.
TDA	475 Contact allergy to cosmetic ingredients	2.3	5 European centres	1996	80 Goossens
TDA	613 Tested with para amino compounds series	10.0	Germany	1995-1999	81 Uter
ALL	**2824**	**Mean: 9.5%**			

Conclusions
Results from several diagnostic patch studies in dermatitis patients show a high rate of contact allergy to toluene-2,5-diamine and toluene-2,5-diamine sulphate. The highest rate was found in dermatitis patients being hairdressers (16.8%, Table 1), followed by dermatitis patients selected due to symptoms or exposure related to cosmetics (9.5%, Table 4), and unselected dermatitis patients (2.4%, Table 3). The rate of contact allergy to toluene-2,5-diamine sulphate in hairdressers (not patients) was 2-2.7% (Table 2).

Due to different selection criteria and different patch test substances used (Table 1-4), conclusions cannot be drawn concerning the trend over time of contact allergy to toluene-2,5-diamine and toluene-2,5-diamine sulphate. The results indicate that patch test reactivity is higher to toluene-2,5-diamine than toluene-2,5-diamine sulphate (Table 1, particularly ref 69 Frosch).

In all publications (except ref Holm), results from patch testing with p-phenylenediamine is given and in several publications also results from tests with additional hair dye substances. In the majority of publications, the rate of contact allergy to p-phenylenediamine was the highest, followed closely by toluene-2,5-diamine, both generally much higher than to other hair dye substances. In some publications, the order between p-phenylenediamine and toluene-2,5-diamine was reversed.

The results do not allow further conclusions concerning concomitant patch test reactions - whether they were the result of multiple sensitisation, or if the result of cross-reactivity to different compounds was due to chemical similarity. Conclusions concerning cross-reactivity require animal studies where induction and elicitation are controlled.

Overall Conclusion Sensitisation
Toluene-2,5-diamine is a well recognised contact sensitizer in humans from both occupational and consumer exposure. Although an EC3 value of 0.31% from a LLNA assay suggests that it is a strong allergen, results from the GPMT indicates an extreme allergen. This latter classification is used in the Opinion.

3.3.4. Dermal / percutaneous absorption

Submission III, 2010

In Vitro Percutaneous Absorption of toluene-2,5-diamine sulfate

Guideline: OECD guideline 428 (2004)
Tissue: Human skin (abdomen, breast, or back; thickness: ca 400 µm)
Number of chambers: 18 from 3 donors for each dose formulation
Method: Automated Teflon flow-through diffusion cells (0.64 cm² exposed area)
Integrity: Tritiated water
Test substance: 1) Hair dye Color Cream formulation containing 0.25, 0.8, 2.4, or 7.2% toluene-2,5-diamine sulfate;

	2) Hair dye Color Cream formulation containing 0.5, 1.6, 4.8, or 14.4% toluene-2,5-diamine sulfate and 0.25, 0.793, 2.38, or 7.135% m-aminophenol mixed 1:1 with a solution containing 6% hydrogen peroxide prior to application.
Batch:	2346 (toluene-2,5-diamine sulfate) DTF0440082CF00 (for formulations)
Purity:	>99.5% HPLC
Radiolabel Batch:	CFQ40199 batch 1: 2,5-diamino[ring-U-14C]-toluene sulfate 72 mCi/mmol 2346: (non-labelled toluene-2,5-diamine sulfate)
Purity:	Radiochemical purity (HPLC): 99.0% 99.7 area% at 290 nm (non-labelled compound)
Area Dosed:	20 mg formulation/cm²
Receptor fluid:	Saline (0.9% NaCl) with 0.01% sodium azide
Solubility:	10 g/l in water
Stability:	8 days
Analysis	Liquid Scintillation Counting
GLP:	In compliance
Date	October 2008

The skin absorption of toluene-2,5-diamine sulfate at concentrations of 0.25, 0.8, 2.4, or 7.2% was investigated with human skin (abdomen, breast, back, thickness ca 400 µm). An area dose of 20 mg/cm² of the final formulation was applied once to the skin (0.64 cm²) in a commercial oxidative hair dye formulation in either the presence or absence of hydrogen peroxide for 30 min.

Automated PTFE flow-through chambers were used. The receptor fluid (0.9% sodium chloride (w/v) containing 0.01% sodium azide, pH ca 6.5) was pumped through the receptor chamber at a rate of 1.6 ml/h. Eighteen chambers were investigated per formulation.

Thirty minutes after substance application, the test item was removed by washing the skin ten times with 0.32 ml water, then once with 0.32 ml washing solution (2% (v/v) sodium dodecyl sulfate) and again ten times with 0.32 ml water. The skin surface was dried with three cotton swabs. Cotton swabs were pooled per skin membrane and extracted with ethanol for at least 24 h. Receptor fluid was collected during the following intervals: 0-0.5 h, 0.5-1 hr, 1-2 h, followed by 2 h intervals until 24 h after application. After 24 h, the diffusion cells were dismantled and the receptor and donor compartments were washed twice with 1 ml ethanol. Each skin membrane was tape stripped 15 times. Skin membranes were digested in a 1.5 M KOH solution with 20% ethanol for at least 24 h.

Ultima GoldTM scintillation liquid was added to samples of the receptor fluid, the diffusion cell washes, the pooled cotton swab extracts, individual tape strips, and digested skin membrane, and radioactivity was determined by liquid scintillation counting.

Results and Discussion
The mean recovery of [^{14}C]-toluene-2,5-diamine sulfate ranged from 99.18 + 3.21% to 102.80 + 5.05% of the applied dose across the eight test groups.

The results of the study provided below are the mean values of 18 samples from 3 donors per formulation tested:

0.25% toluene-2,5-diamine sulfate

	non-oxidative	oxidative

Amount in	µg/cm² skin surface	% of applied dose	µg/cm² skin surface	% of applied dose
Applied formulation	47.9 ± 0.4	100 ± 0.8	60.9 ± 9.3	100 ± 15.3
Skin wash	47.03 ± 2.54	98.13 ± 5.20	56.61 ± 9.66	92.69 ± 3.77
Stratum corneum	0.17 ± 0.03	0.36 ± 0.07	1.60 ± 0.32	2.72 ± 0.86
Skin	0.19 ± 0.10	0.40 ± 0.21	0.79 ± 0.43	1.32 ± 0.77
Receptor fluid	1.55 ± 0.47	3.24 ± 0.98	1.29 ± 0.26	2.15 ± 0.51
Total recovery	48.98 ± 2.40	102.18 ± 4.93	60.47 ± 9.61	99.18 ± 3.21
Total absorption*	**1.75 ± 0.52**	**3.65 ± 1.08**	**1.83 ± 0.46**	**3.49 ± 1.02**

0.8% toluene-2,5-diamine sulfate

	non-oxidative		oxidative	
Amount in	µg/cm² skin surface	% of applied dose	µg/cm² skin surface	% of applied dose
Applied formulation	173.4 ± 23.9	100 ± 13.8	181.5 ± 31.2	100 ± 17.2
Skin wash	159.10 ± 21.80	91.94 ± 5.54	171.48 ± 32.18	94.25 ± 3.13
Stratum corneum	1.48 ± 0.64	0.89 ± 0.46	3.59 ± 1.40	1.98 ± 0.68
Skin	2.16 ± 0.89	1.25 ± 0.49	2.28 ± 1.33	1.23 ± 0.61
Receptor fluid	14.09 ± 2.78	8.29 ± 2.06	4.61 ± 1.36	2.64 ± 0.91
Total recovery	177.54 ± 20.45	102.80 ± 5.05	182.30 ± 32.93	100.30 ± 2.48
Total absorption*	**16.27 ± 2.94**	**9.55 ± 2.17**	**6.92 ± 1.56**	**3.88 ± 0.92**

2.4% toluene-2,5-diamine sulfate

	non-oxidative		oxidative	
Amount in	µg/cm² skin surface	% of applied dose	µg/cm² skin surface	% of applied dose
Applied formulation	514.2 ± 66.7	100 ± 13.0	566.9 ± 83.7	100 ± 14.8
Skin wash	463.15 ± 66.67	89.98 ± 3.59	548.56 ± 89.60	96.62 ± 3.73
Stratum corneum	3.55 ± 1.87	0.69 ± 0.36	9.64 ± 3.94	1.78 ± 0.93
Skin	5.30 ± 2.20	1.05 ± 0.47	5.81 ± 2.42	1.07 ± 0.55
Receptor fluid	41.87 ± 11.66	8.19 ± 2.15	15.96 ± 4.52	2.83 ± 0.79
Total recovery	515.64 ± 68.90	100.25 ± 1.84	581.01 ± 88.22	102.49 ± 3.06
Total absorption*	**47.23 ± 13.35**	**9.25 ± 2.54**	**21.81 ± 4.87**	**3.90 ± 0.93**

7.2% toluene-2,5-diamine sulfate

	non-oxidative		oxidative	
Amount in	µg/cm² skin surface	% of applied dose	µg/cm² skin surface	% of applied dose
Applied formulation	1599.1 ± 285.6	100 ± 17.9	1574.4 ± 303.6	100 ± 19.3
Skin wash	1497.17 ± 278.24	93.58 ± 2.94	1483.91 ± 295.61	94.19 ± 3.79
Stratum corneum	9.03 ± 3.48	0.57 ± 0.20	32.62 ± 10.95	2.12 ± 0.70
Skin	10.06 ± 3.79	0.63 ± 0.21	11.25 ± 6.42	0.71 ± 0.36
Receptor fluid	90.82 ± 33.76	5.79 ± 2.20	42.63 ± 11.23	2.76 ± 0.75
Total recovery	1616.29 ± 284.15	101.14 ± 1.27	1577.34 ± 302.13	100.26 ± 3.37
Total absorption*	**101.03 ± 36.67**	**6.43 ± 2.36**	**54.03 ± 15.38**	**3.49 ± 0.92**

* Total absorption is defined as the amount in the receptor fluid, the receptor compartment wash and skin membrane (epidermis and dermis), excluding tape strips.

Under both oxidative and non-oxidative conditions, a linear increase in the amount of toluene-2,5-diamine sulfate in the receptor fluid and the total amount absorbed (receptor fluid plus skin (epidermis plus dermis) was observed. Under oxidative conditions, the amount of radioactivity recovered from the tape strips (i.e., stratum corneum) was considerably higher when compared to non-oxidative conditions, whereas the total amount absorbed was lower under oxidative conditions when compared to non-oxidative conditions with the exception of the lowest concentration tested (0.25% toluene-2,5-diamine sulfate).

Ref.: 11 (subm III)

Comment
These were well performed studies. Therefore, mean + 1SD may be used as the amount absorbed in calculating the MOS. Experiments were conducted with toluene-2,5-diamine sulfate in a range of dilutions from 0.25% to 7.2%.
For 2.4% toluene-2,5-diamine sulfate, the amount absorbed under non-oxidative conditions (mean + 1SD) is 47.23 + 13.35 = 60.58 µg/cm². Under oxidative conditions, the amount absorbed is 21.81 +4.87 = 26.68 µg/cm². The latter value may be used for the conventional MOS calculation after correction (26.68 x 3.6 / 2.4 = 40.02 µg/cm²) since the intended on head concentration is 3.6%.

Taken from SCCP/1084/07

Percutaneous absorption/penetration in-vitro, human skin

Guideline:	/
Tissue:	Human abdominal skin (thickness 350 µm) from women, 3 donors
Method:	Dynamic diffusion cells, surface area of application 2.0 cm^2
Test substance:	Formulation 175307/Water: *toluene-2,5-diamine sulfate* (**4.5%** final applied concentration) in a hair dye formulation mixed with water prior to application Formulation 175307/peroxide: *toluene-2,5-diamine sulfate* (**4.5%** final applied concentration) in a hair dye formulation mixed with hydrogen peroxide prior to application Formulation 175308/peroxide: *toluene-2,5-diamine sulfate* (**4.5%** final applied concentration) in a hair dye formulation containing m-aminophenol coupler and mixed with hydrogen peroxide prior to application
Batch:	CFQ9920 (^{14}C-labelled substance) CH1134 (non-labelled substance)
Purity:	Radiochemical purity: 97.2% (HPLC) Non-labelled substance: 99.7% (titrated); o-toluidine < 100 ppm (HPLC)
Dose levels:	0.9 mg *toluene-2,5-diamine sulfate* / cm^2
Dosing schedule:	30 min application
Replicates:	Two different *in vitro* systems were used: dermatomed human skin (8 replicates) and isolated human epidermis (8 replicates)
Study year:	1997
GLP:	not in compliance

[^{14}C]-*Toluene-2,5-diamine sulfate* and non-labelled substance were added to a final concentration of 9% to the hair dye bases no. 175307 and no. 175308. The formulation no. 175308 contained an equimolar amount of the coupler m-aminophenol.

Three different mixtures were then prepared:

1. formulation no. 175307 mixed with water (50:50)
2. formulation no. 175307 mixed with hydrogen peroxide (50:50)
3. formulation no. 175308 mixed with hydrogen peroxide (50:50) and coupler m-aminophenol.

These mixtures were applied in amounts of 20 mg/cm^2 (equals 0.9 mg toluene-2,5-diamine sulfate/cm^2) to the surface of either human skin samples of 350 µm thickness or samples of isolated epidermis mounted in perfusion cells with 2 cm^2 application area. For each *in vitro* model, 8 perfusion cells were initially prepared and a skin integrity test was performed. After 30 min the test substance was rinsed off with water and sodium lauryl sulfate solution. The intradermal distribution, the amounts of penetrated substance and the kinetics of the dermal penetration of [^{14}C]-toluene-2,5-diamine sulfate were analysed over a period of 24 h. Results for the dermatomed skin are presented below.

Results
The number of cells which could be used for data analysis was 7, 6 and 4 respectively for the three formulations using isolated skin samples and 8, 7 and 7 respectively for the dermatomed skin.

The majority of the test substance (93.5% of the applied dose) was recovered from the skin by rinsing 30 min after application. The cumulative penetration, based on the amount of radioactivity in the receptor fluid, approached a plateau at approximately 5 hours. The presence of peroxide diminished the absorbed amount of radioactivity while the amount adsorbed in the stratum corneum was increased. As a consequence the sum of the amounts of radioactivity in the total skin and in the receptor fluid was about the same for the three formulations.

The systemically available amount of [^{14}C]-toluene-2,5-diamine sulfate was calculated as the amount found in the receptor fluid plus the residual amount in the skin, excluding the amount adsorbed to the stratum corneum. The results for the dermatomed skin samples were as follows:

	Total absorption	
	µg$_{eq}$/cm^2	**% of applied dose**
Formulation 175307/water	40.31 ± 23.77 (19.48-78.03)	4.17 ± 2.54 (2.04-8.56)
Formulation 175307/peroxide	32.03 ± 26.58 (12.54-76.04)	3.44 ± 2.84 (1.29-8.12)
Formulation 175308/peroxide/coupler	31.26 ± 11.64 (16.54-51.82)	3.41 ± 1.32 (1.88-6.07)

Ref.: 28

Percutaneous absorption/penetration in vitro, pig skin (study 1)

Guideline: OECD Draft Guideline - Skin absorption: *in vitro* method (2000)
Tissue: Porcine skin (thickness: 560-750 µm); 2 donors, 1 male, 1 female
Test substance: Formulation A: toluene-2,5-diamine sulfate (5.4% final applied concentration) in a hair dye formulation containing resorcinol coupler; mixed with water prior to application
Formulation B: 5.4% toluene-2,5-diamine sulfate (5.4% final applied concentration) in a hair dye formulation containing coupler; mixed with a solution of hydrogen peroxide (3% final concentration) prior to application
Formulation C: toluene-2,5-diamine sulfate (5.4%) in an aqueous solution
Method: Static diffusion cells, surface area of application 1.0 cm^2
Batch: 3362-259 [Ring ^{14}C (U)]-toluene-2,5-diamine sulfate 1.49 GBq/mmol (40.37 mCi/mmol)

	46847 (non-labelled toluene-2,5-diamine sulfate)
Purity:	Radiochemical purity (HPLC): 99.3% 99.9% (non-labelled substance)
Dose Applied:	1.08 mg toluene-2,5-diamine sulfate/cm^2 (20 mg of formulation)
Replicates:	2 experiments, 6 replicates for each pig for each experimental group in each experiment
GLP:	In compliance

The percutaneous penetration of toluene-2,5-diamine sulfate at a concentration of 5.4%, below the maximum concentration intended for hair colorants, was investigated with pig skin prepared from animals and stored at -20 °C until use.

The test substance was applied in a formulation containing resorcinol (coupler) which was mixed with either water or hydrogen peroxide (final concentration 3%) prior to application. A preparation of 5.4% toluene-2,5-diamine sulfate in water was also tested. Skin integrity was determined at the start and at termination by measurement of the transdermal electrical resistance.
20 mg of the respective formulation (corresponding to 1.08 mg toluene-2,5-diamine sulfate and ca. 0.5 MBq/cm^2) was applied to the skin surface, and rinsed off after 30 minutes. Samples of the receptor fluid were taken before application of the test substance and after 0.5 h, 1 h, 2 h, 4 h, 6 h, 24 h, 29 h, 48 h. At the end of the experiment the skin was rinsed, and the stratum corneum was stripped with adhesive tape. Radioactivity in the receptor fluid, skin, tapes, and rinsings was measured by liquid scintillation counting.

Results
The majority of the test substance (86.9-95.8% of the applied dose) was recovered from the skin by rinsing 30 min after application. The cumulative penetration, based on the amount of radioactivity in the receptor fluid, approached a plateau at approximately 24 hours. Percutaneous absorption was calculated by adding the amounts of radioactivity measured in epidermis to those in dermis and in receptor fluid (see table below). The total recovery was 98.4%, 94.4%, and 96.2% for Formulations A, B, and C, respectively.

5.4% final			Total absorption	
	Receptor Fluid ($\mu g_{eq}/cm^2$)	Epidermis plus dermis ($\mu g_{eq}/cm^2$)	$\mu g_{eq}/cm^2$	% of applied dose
Formulation A (with coupler + water)	10.7	10.1	20.84 ± 5.1 (13.84–32.06)	1.72 ± 0.32 (1.23-2.44)
Formulation B (with coupler + peroxide	14.9	13.5 $\mu g_{eq}/cm^2$	28.46 ± 9.5 (15.68- 44.90)	2.39 ± 0.79 (1.25-3.88)
Formulation C (aqueous vehicle)*	13.3	28.6 $\mu g_{eq}/cm^2$	41.98 ± 19.9 (18.20-75.99)	3.94 ± 2.07 (1.60-8.27)

* The higher values for epidermis plus dermis for Formulation C were attributed to a methodological problem with the first experiment run with this formulation.

Ref.: 29

Dermal absorption / penetration in vitro, pig skin (study 2)

Guideline:	OECD Draft Guideline – Skin absorption: *in vitro* method (2000)
Tissue:	Porcine back skin (thickness: 0.9 ± 0.1 mm); 1 female donor
Method:	Dynamic Teflon diffusion chambers, surface area of application 4.0 cm^2
Test substance:	4.6% toluene-2,5-diamine sulfate in a hair dye formulation not containing coupler or hydrogen peroxide
Batch:	CFQ13783 batch1 [ring-^{14}C (U)]-toluene-2,5-diamine sulfate (55 mCi/mmol) R0017930 (Ondal) non-labelled toluene-2,5-diamine sulfate
Purity:	[ring-^{14}C (U)]-toluene-2,5-diamine sulfate HPLC: 98.2 area% (254 nm)

Non-labelled-toluene-2,5-diamine sulfate HPLC: 99.9 area% (254 nm); 99.7 weight% by NMR
Dose applied: 4.6 mg toluene-2,5-diamine sulfate / cm^2 (100 mg of formulation)
Replicates: 6
GLP: In compliance

Dermatomed skin preparations of ~1000 µm thickness were mounted into Teflon diffusion cells with an application area of 4 cm^2. An integrity test using tritiated water was performed before the skin samples were covered with the test substance (400 mg of the hair dye formulation containing 4.6% of toluene-2,5-diamine sulfate per 4 cm^2). After 30 min the formulation was removed by intensive washing and fractions of the receptor fluid were collected after 16 h, 24 h, 40 h, 48 h, 64 h and 72 h. Epidermis and upper dermis were separated by heat treatment and extracted for analysis.

Results
The majority of the test substance (92.8%) was removed from the skin by rinsing 30 min after application. The cumulative penetration, based on the amount of radioactivity in the receptor fluid, approached a plateau at approximately 24 hours. Percutaneous absorption was calculated by summing the cumulative amounts of substance measured in the receptor fluid and upper dermis. The results were as follows:

Receptor Fluid	Upper dermis	Total absorption
5.6 ± 1.7 (4.0-8.2)	4.9 ± 1.9 (3.3-8.0)	10.5 ± 3.2 (0.2% of applied dose) A_{max} 16.2 (0.4% of applied dose)

There was no tape stripping of the skin to remove stratum corneum from the epidermis, and epidermis was not included in the calculation of total percutaneous absorption. The amount present in the epidermis corresponded to 7.0 ± 1.4 $\mu g_{eq}/cm^2$. Total recovery of radioactivity was 93.2%.

Ref.: 30

Comment
The test formulation contained only 4.6% toluene-2,5-diamine sulfate where the maximum in-use concentration is 7.2%. The amount of formulation applied (100 mg/cm²) is not in accordance with the SCCP Notes of Guidance (20 mg formulation/cm²).

Dermal absorption / penetration in vitro, pig skin (study 3)

Guideline: OECD Draft guideline: Skin absorption: *in vitro* method (1999)
Tissue: Porcine skin (thickness: 1 mm); 1 male donor
Method: Dynamic diffusion chambers, surface area of application 0.785 cm²
Test substance: 0.6% 2-methyl-1,4-benzenediamine sulphate in acetone/H_2O/olive oil (2:2:1)
Batch: R0054969
Purity: > 99% (HPLC)
Dose applied: 0.59 mg 2-methyl-1,4-benzenediamine sulphate /cm^2 (78µl of solution applied)
Replicates: 6 (5 used for analysis)
GLP: In compliance

The objective of this study was to examine dermal penetration from the vehicle used in a Local Lymph Node Assay (LLNA).

A solution of 2-methyl-1,4-benzenediamine sulphate (corresponding to 0.59 mg/cm²) in acetone/H_2O/olive oil (2:2:1) was applied to the skin samples and rinsed off after 30 min

using water and a shampoo. The receptor fluid was sampled after 16, 24, 40, 48, 64 and 72 h and analysed for 2-methyl-1,4-benzenediamine sulphate by HPLC. Subsequently upper skin (stratum corneum + upper *stratum germinativum*) and lower skin (lower *stratum germinativum* + upper dermis) were separated by heat treatment. Extracts of skin layers were prepared and analysed by HPLC.

Results
The majority of the test substance (>100%) was removed from the skin by rinsing 30 min after application. Total recovery was 107.7%. The cumulative penetration, based on the amount of radioactivity in the receptor fluid, had reached a plateau at 16 hours. Percutaneous absorption was calculated by summing the cumulative amounts of substance measured in the receptor fluid and upper dermis. The results were as follows:

Receptor Fluid $\mu g_{eq}/cm^2$	**Lower skin (lower *stratum germinativum* + upper dermis) $\mu g_{eq}/cm^2$**	**Total absorption $\mu g_{eq}/cm^2$**
24.7 ± 7.2 (18.8-36.9)	7.2 ± 3.3 (3.94-12.64)	31.9 (5.35% of applied dose) A_{max} 42.2 (7.08% of applied dose)

Ref.: 31

Overview of the *in vitro* percutaneous absorption studies that have been performed with t*oluene-2,5-diamine sulfate:*

Studies from submission II		
Human skin	^{14}C-labelled	Formulation (4.5%) without coupler; mixed with water Formulation (4.5%) without coupler; mixed with hydrogen peroxide Formulation (4.5%) containing coupler (m-aminophenol); mixed with peroxide
Pig skin (study 1)	^{14}C-labelled	Formulation (5.4%) with coupler (resorcinol); mixed with water Formulation (5.4%) with coupler (resorcinol); mixed with hydrogen peroxide Aqueous solution (5.4%)
Pig skin (study 2)	^{14}C-labelled	Formulation without coupler or hydrogen peroxide
Pig skin (study 3)	Non-labelled	0.6% solution of 2-methyl-1,4-benzenediamine sulphate in acetone/water/olive oil
Studies from submission III		
Human skin	^{14}C-labelled	Formulation containing 0.25, 0.8, 2.4, or 7.2% toluene-2,5-diamine sulfate without coupler Formulation containing 0.5, 1.6, 4.8, or 14.4% toluene-2,5-diamine sulfate containing coupler (m-aminophenol); mixed with peroxide

General conclusion on percutaneous absorption studies *in vitro*
Percutaneous absorption studies have been conducted in both human skin and pig skin *in vitro.*
In the first study with human skin (submission II), using formulations containing 4.5% toluene-2,5-diamine sulfate + coupler + hydrogen peroxide, the percutaneous absorption was 31.26 ± 11.64 (A_{max} 51.82) $\mu g_{eq}/cm^2$ (3.41 ± 1.32 (A_{max} 6.07) % of applied dose). The value of 51.82 $\mu g_{eq}/cm^2$ (A_{max}, formulation + hydrogen peroxide + coupler) after extrapolation to 7.2% concentration (51.82 x 7.2/4.5 = 82.9 $\mu g_{eq}/cm^2$) was used for the calculation of the Margin of Safety in the previous opinion SCCP/1084/07.

Studies with pig skin employed various application conditions (e.g., formulations containing coupler with or without hydrogen peroxide, formulations without coupler and peroxide, aqueous solution, or acetone/water/olive oil solution). The percutaneous absorption values for formulations containing 4.6 - 5.4% toluene-2,5-diamine sulfate (including studies of formulations with and without coupler + peroxide) ranged from 10.5 to 28.5 $\mu g_{eq}/cm^2$ (0.2 to 2.39% of applied dose). The study involving 0.6% toluene-2,5-diamine sulfate in an acetone/water/olive oil vehicle showed the greatest penetration (31.9 (A_{max} 42.2) $\mu g_{eq}/cm^2$ or 5.35 (A_{max} 7.08) % of applied dose), including the greatest amount present in the receptor fluid, but the application vehicle was not considered representative of human use conditions. These results did however confirm the adequacy of the vehicle used in the LLNA.

The percutaneous absorption values obtained in these *in vitro* experiments were comparable to those calculated from a study conducted in human volunteers with a ^{14}C-toluene-2,5-diamine sulfate-containing hair dye (20 or 71 $\mu g_{eq}/cm^2$ see Section 3.3.9.2.).

The data obtained in the different percutaneous absorption studies vary in the range of approximately 10 to 70 µg/cm². Such a variation is expected in view of the differences in design and data evaluation across studies. Factors which influenced the results were related to the test formulation (test substance concentration, presence of hydrogen peroxide and reaction partner, vehicle) to test model details (species/source and skin type) and to the number and type of compartments included in the calculation of systemic exposure.

In submission III of 2010 an additional well performed dermal absorption study with human skin *in vitro* was included. From this study the mean + 1SD may be used as the amount absorbed. The intended use of toluene-2,5-diamine was at a maximum of 4% 'on head' (7.2% calculated as sulphate salt). Experiments with toluene-2,5-diamine sulfate were conducted in a range of dilutions from 0.25% to 7.2%. For 7.2% toluene-2,5-diamine sulfate, the amount absorbed under non-oxidative conditions (mean + 1SD) was 137.7 µg/cm². Under oxidative conditions, the amount absorbed is 69.4 µg/cm², which was used for MOS calculation in a previous opinion (SCCS/1390/11).

For 2.4% toluene-2,5-diamine sulfate, the amount absorbed under non-oxidative conditions (mean + 1SD) is 47.23 + 13.35 = 60.58 µg/cm². Under oxidative conditions, the amount absorbed is 21.81 + 4.87 = 26.68 µg/cm². The latter value may be used for the conventional MoS calculation after correction (26.68 x 3.6 / 2.4 = 40.02 µg/cm²) since the intended on head concentration is 3.6%.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (14 days) oral / dermal / inhalation toxicity

Range finding study

Guideline:	/
Species/strain:	Sprague-Dawley rats, Crl:CD(SD)BR
Group size:	10 animals per sex per dose
Test substance:	toluene-2,5-diamine sulfate in deionised water
Batch:	CH 1143
Purity:	99.7%
Dose:	0, 7.5, 15, 30, 60 mg/kg bw/d
Route:	oral, gavage
Exposure:	once daily for 14 d
GLP:	in compliance

Doses of 0, 7.5, 15, 30, 60 mg/kg bw/d of toluene-2,5-diamine sulfate in deionised water were given once daily to 10 rats of each sex. Animals were observed twice daily for mortality and daily for clinical signs, body weights and food intake were recorded weekly. At

the end of treatment period blood samples for the investigation of haematology and biochemistry were taken, the animals were sacrificed and subjected to necropsy, organs were weighed and tissues were examined microscopically.

Results
One animal died after blood withdrawal which was not associated with the test substance. No treatment related clinical observations were recorded and body weight and food intake were not changed. While no haematological parameters were altered several biochemistry parameters were influenced at and above 30 mg/kg bw/d (AST, CPK, LDH, ALT (only at 60 mg/kg bw/d)). The mean absolute and relative (to body weight) liver weights of both sexes at 60 and males only at 30 mg/kg bw/d were increased. At necropsy no macroscopic abnormalities were noted. Myocyte degeneration was noted in the heart, skeletal muscle, tongue and diaphragm in both sexes in all dose groups.

Ref.: 32

Peer review of two external experts
The histological slides from relevant tissues (diaphragm, skeletal muscle, tongue and heart) from this study were reviewed by two independent pathologists. Both pathologists agreed that treatment-related myofiber necrosis, degeneration, and/or inflammatory change were present in skeletal muscle, tongue, and diaphragm of both males and females given 30 or 60 mg/kg bw/d in this study and that the NOAEL for muscle degenerative change in this study was 15 mg/kg bw/d.

Ref.: 4, 5 (subm III)

3.3.5.2. Sub-chronic (90 days) oral / dermal / inhalation toxicity

Study 1

Guideline:	OECD 408 (1981)
Species/strain:	Sprague-Dawley rats, Crl:CD(SD)BR
Group size:	15 animals per sex per dose
Test substance:	toluene-2,5-diamine sulfate in deionised water
Batch:	CH 1143
Purity:	99.7%
Dose:	0, 2.5, 5, 10, 20 mg/kg bw/d
Route:	oral, gavage
Exposure:	once daily for 13 weeks
GLP:	in compliance

Doses of 0, 2.5, 5, 10, 20 mg/kg bw/d of toluene-2,5-diamine sulfate in deionised water were given once daily to 15 rats of each sex for 13 weeks. Animals were observed daily for mortality and clinical signs, body weights and food intake were recorded weekly. Ophthalmoscopy was performed on all animals before the start of treatment and during week 13. Blood and urine samples were taken during weeks 4 and 12/13.

At the end of treatment period the animals were sacrificed and subjected to necropsy, organs were weighed and tissues were examined microscopically.

Results
While 2 males were killed *in extremis* no treatment related deaths were observed. No treatment-related clinical signs were observed. Body weights and body weight gains as well as food consumption were not affected by treatment. Changes in haematology were not considered to be test substance related. In blood chemistry significant increases in AST levels were seen in females from 5 mg/kg bw/d upwards and urinalysis revealed increased urine levels associated with decreases in specific gravity at 10 (females) and 20 mg/kg bw/d (males and females). Ophthalmoscopic examination revealed retinal hyper-reflectivity in 2 males given 20 mg/kg bw/d, 1 male given 2.5 mg/kg bw/d and 1 female given 5 mg/kg

bw/d. During histopathology retinal degeneration was diagnosed only for the males. The results were re-evaluated in a pathology peer review and it was concluded that this linear focal retinopathy was similar to the spontaneous incidence of focal linear degeneration of around 3% in this rat strain. No dose-response relationship was seen. At 20 mg/kg bw/d an increased incidence of abnormally shaped pituitary glands was observed.

Conclusion
The NOAEL is considered to be 2.5 mg/kg bw/d (free base: 1.4 mg/kg bw/d), based on an increase in AST levels.

Ref.: 33

Comment of the SCCP
The myocyte degeneration observed in the dose range finding study was not reported in the main study. Both evaluations were made by the same evaluator in the same time period. No comment on these conflicting results was given in discussion of the study results as well as in the dossier.
A further 12-week oral toxicity study in rats was cited in Ref. 52 and also referenced in the dossier (Ref. 93) but not provided to the SCCP. This study should be checked with regard to myopathies.

Peer review of two external experts
The pathology experts reviewed the clinical chemistry data from this 90-day study in order to clarify the toxicological relevance of those findings. Both pathologists agreed that the minimal elevations in AST concentration observed in females, but not in males, at week 4 at doses of 5, 10, and 20 mg/kg bw/d in the 90-day study were not related to treatment. These changes were not dose-related, of minimal magnitude (not higher than 1.1-fold the mean control value), and individual values were essentially within the historical control range. No elevation in AST concentration was observed at the end of the study in males or females at doses of up to 10 mg/kg bw/d. At 20 mg/kg bw/d, mean AST concentrations were elevated in males and females at the end of the study as some animals clearly exceeded historical control ranges. One of the reviewers noted, in contrast to the original evaluation and the second reviewer, muscle degeneration in multiple organs at 20 mg/kg bw/d. Considering the AST results as well as other clinical pathology results, both pathologists concluded that the NOAEL for clinical pathology was 10 mg/kg bw/d.

Comment of the SCCS
AST (aspartate aminotransferase, also known as glutamate oxalacetate transaminase, GOT) release is closely related to myotoxicity and, therefore, changes in the plasma level have to be considered with a substance known to induce myodegenerative changes. It is well known that p-phenylenediamine and several derivatives including toluene-2,5-diamine induce such effects on skeletal muscles. Toluene-2,5-diamine was effective already after a s.c. 3-day treatment with 22 mg/kg bw/d in rats (e.g. Munday et al. 1990; Munday, Manns 1999). However, the slightly increased values observed at week 4 in the dose groups 5, 10, and 20 mg/kg bw (89, 89, 87) were not dose-related and well within historical controls (61-105). In contrast, at 20 mg/kg bw/d in week 13 the figure (135) was clearly exceeding controls and according to the one peer reviewer also degenerative changes were seen at this dose.

Ref.:AR1, AR2

Study 2 (Submission III, 2010)

Guideline:	OECD Guideline 408 (1981)
Species/strain:	Sprague Dawley Rats, Crl:CD(SD)
Group size:	15 animals per sex per dose level plus 5 animals per sex per dose level for 28-day recovery in control and 20 mg/kg bw/d groups
Test substance:	toluene-2,5-diamine sulfate
Batch:	01057257RB-03

Purity:	99.8%
Vehicle:	Reverse osmosis deionized water, pH 5.0 ± 0.3
Dose levels:	0, 2.5, 5, 10, and 20 mg/kg bw/d
Dose volume:	10 ml/kg bw
Route:	Oral, gavage
Exposure period:	Once daily over 91 days, followed by a 28-day recovery period (control and 20 mg/kg bw/d groups only)
Date:	07/2009 – 08/2010 (performed outside the EU)
GLP:	In compliance

Doses of 0 (control group), 2.5, 5, 10, and 20 mg/kg bw/d of toluene-2,5-diamine sulfate dissolved in vehicle (reverse osmosis deionized water, pH 5.0 ± 0.3) were administered once daily to groups of 15 male and 15 female Sprague Dawley rats by oral gavage in a total volume of 10 ml/kg bw for a periods of 91 consecutive days followed by a 28-day recovery period (control and 20 mg/kg bw/d groups only). The control group received vehicle only. Dose formulations were prepared daily. Animals were housed individually.

Dose levels were selected on the basis of a previously conducted 13 week oral toxicity study included in Submission II (Ref. 33). A NOAEL was considered by the SCCP to be 2.5 mg/kg bw/d (free base: 1.4 mg/kg bw/d), based on an increase in AST levels and conflicting results on myocyte degeneration observed in the dose range finding and the main study (SCCP/1084/07).

In-life evaluations included twice daily mortality/morbidity checks, daily recording of clinical signs, weekly recording of clinical observations, body weight and food consumption, abbreviated functional observational battery (FOB) (days 26 and 54) and full FOB (days 89-90), and ophthalmology examination during week 13. Blood and urine were collected for clinical pathology at the end of week 13 and at the end of the recovery period. All animals were subjected to a complete gross necropsy examination. Organ weights were measured and tissues were taken from animals in all treatment groups and recovery groups and preserved in 10% neutral buffered formalin. Histopathological investigation was performed on all collected tissues from the animals in the control, high dose, and recovery group. The skeletal muscle (thigh and diaphragm), tongue, heart, and periocular muscle were identified as possible target tissues in the high dose treatment group (20 mg/kg bw/d), and therefore, these tissues were processed and examined from the other treatment groups (2.5, 5, and 10 mg/kg bw/d).

Results
Dose formulations prepared and analyzed on Days 1, 8, 50, and 85 verified that the target concentrations were achieved (± 15%).

No treatment-related mortality occurred and there were no adverse treatment-related effects on clinical signs, functional observational battery findings, body weights, body weight changes, food consumption, ophthalmic findings, haematology parameters, coagulation parameters, urinalysis parameters, gross necropsy findings, or organ weights.

The only treatment-related clinical chemistry change was an elevation in aspartate aminotransferase (AST) at both males and females at 20 mg/kg bw/d, although the increase in the mean value in males was not statistically significant and substantial elevations were observed in only 4/15 animals whereas in females 12/15 AST values were substantially elevated. The elevation in AST correlated with microscopic changes in skeletal muscle. At the end of the 28-day recovery period, AST levels in 20 mg/kg bw/d animals were comparable to controls.

Treatment-related microscopic changes were observed in males and females at 20 mg/kg bw/d and were found in skeletal muscle of the thigh, diaphragm, and tongue and in the periocular muscle of the eye. These changes consisted of mononuclear cell infiltrates (eye,

diaphragm, thigh, and tongue), muscular degeneration (diaphragm, thigh, and tongue), and muscular regeneration (diaphragm). After a 28-day treatment-free period, the treatment-related findings noted at the terminal sacrifice were not present at the recovery group sacrifice.

Conclusion
Administration of toluene-2,5-diamine sulfate once daily by oral gavage at a dose of 20 mg/kg bw/d was associated with microscopic changes in the skeletal muscle of the thigh, diaphragm, tongue, and periocular muscle that correlated with elevations in AST. These target organ effects resolved following the 28-day recovery period. No treatment-related findings were reported at 10 mg/kg bw/d. Based on these results, the no-observed-adverse-effect level (NOAEL) was determined to be 10 mg/kg bw/d.

Ref.: 13 (subm III)

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Taken from SCCP/1084/07

Bacterial Reverse Mutation Test

Guideline:	OECD 471
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537
Replicates:	triplicates in 2 individual experiments both in the presence and absence of S9-mix.
Test substance:	toluene-2,5-diamine sulfate
Solvent:	DMSO
Batch:	R99053665
Purity:	99.2 - 99.8%
Concentrations:	3 - 5000 µg/plate without and with S9-mix
Treatment:	direct plate incorporation with at least 48 h incubation without and with S9-mix
GLP:	in compliance

Toluene-2,5-diamine sulfate was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-experiment for toxicity and mutation induction with all strains and both without and with S9-mix. Toxicity was evaluated for 8 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of revertant colonies and/or clearing of the bacterial background lawn. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in all strains used, the pre-experiment is reported as experiment I. Both experiments were performed with the direct plate incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results
Toxic effects evident as reduction in the number of revertants were observed at 2500 µg/plate and above with the exception of TA100 and TA102 without S9-mix (5000 µg/plate) and TA102 with S9-mix where no toxicity were seen. All incubated plates showed normal background growth up to 5000 µg/plate.

In both experiments in the presence of S9-mix a dose dependent increase in revertant colonies was observed in TA98, TA100, TA1535 and TA1537. In TA102 and in the absence of S9-mix in all five tester strains toluene-2,5-diamine sulfate did not induce a biologically relevant increase in revertant colonies.

Conclusion
Under the experimental conditions used toluene-2,5-diamine sulfate was genotoxic (mutagenic) in this gene mutation test in bacteria.

Ref.: 34

In Vitro* Mammalian Cell Gene Mutation test (*tk* locus*)

Guideline:	OECD 476
Cells:	L5178Y Mouse lymphoma cells
Replicates:	triplicates in 2 independent experiments
Test substance:	A 5 (toluene-2,5-diamine sulfate)
Solvent:	NH_4OH (1%)
Batch:	EFH 290394
Purity:	97.3% (technical product)
Concentrations:	1.0 - 15.0 µg/ml (without S9-mix) 10.0 - 100.0 µg/ml (with S9-mix)
Treatment	4 h treatment without and with S9-mix; expression period 72 h and selection period of 11-13 days
GLP:	in compliance

Toluene-2,5-diamine sulfate was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a pre-test on toxicity measuring relative suspension growth. In the main tests, cells were treated for 4 h followed by an expression period of 72 h to fix the DNA damage into a stable *tk* mutation. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was measured in the main experiments as percentage relative total growth of the treated cultures relative to the total growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-experiment on toxicity distinct toxic effects could be observed with concentrations above 3.0 µg/ml. In both experiments the required toxicity was reached (10-20% survival compared to the concurrent negative controls) without S9-mix. In the presence of S9-mix the required level of toxicity was not achieved.
Occasionally an increase in mutant frequency was observed in both experiments with and without S9-mix. However, these results appeared not reproducible and are, therefore, considered as not biologically relevant.

Conclusion
Under the experimental conditions used, toluene-2,5-diamine sulfate did not induce gene mutations in this gene mutation test in mammalian cells.

Ref.: 35

Comments
The required toxicity (10-20% survival compared to the concurrent negative controls) was not reached in the experiments with S9-mix.

In vitro Chromosome Aberration Test

Guideline:	OECD 473
Replicates:	duplicate cultures
Cells:	V79
Test substance:	SAT 010935 (toluene-2,5-diamine sulfate)
Solvent:	culture medium (Minimum essential medium, MEM)
Batch:	46847
Purity:	99.9%
Concentrations:	2.5, 5.0 and 10.0 µg/ml without S9-mix 100, 200, 300 and 400 µg/ml with S9-mix
Treatment:	4 h treatment and harvest time 18 after start of treatment both in the absence and presence of S9-mix
GLP:	in compliance

Toluene-2,5-diamine sulfate was investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in V79 cells. Test concentrations were based on the results of a range finding pre-test on cell number and cell morphology with 4 h and 24 h treatment. The highest dose in the pre-test was the prescribed maximum concentration (2210 µg/ml ≈ 10 mM). Cells were treated for 4 h and harvested 18 h after the start of treatment. 2.5 h before harvest, each culture was treated with colcemid (final concentration 0.2 µg/ml) to block cells at metaphase of mitosis. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Toxicity was determined by measuring the decrease in the mitotic index. Chromosome (metaphase) preparations were stained with Giemsa and examined microscopically for chromosomal aberrations and the mitotic index. Negative and positive controls were in accordance with the OECD draft guideline.

Results
In the pre-test, precipitation was observed at doses far above the test doses of the main test. No relevant influence of toluene-2,5-diamine sulfate on the osmolarity was observed. A slight pH shift was adjusted with NaOH.
Biologically relevant increases in polyploid metaphases were not found. At the concentrations evaluated, no clear toxic effects indicated by reduced mitotic indices or reduced cell numbers were found. The required 50% decrease in MI compared to the concurrent control at the highest dose tested was not reached.
Both in the absence and presence of S9-mix toluene-2,5-diamine sulfate induced a more or less dose dependent and biologically relevant increase in cells with chromosomal aberrations.

Conclusion
Under the experimental conditions used the increase in cells with structural chromosomal aberrations indicates a genotoxic (clastogenic) activity of toluene-2,5-diamine sulfate in V79 cells *in vitro*.

Ref.: 36

In vitro unscheduled DNA synthesis test

Guideline:	/
Cells:	hepatocytes from male Sprague-Dawley caesarean-derived rats (Crl:COBS®(SD)BR) or Golden Syrian hamsters (LAK: LVG(SYR))
Replicates:	2 independent experiments
Test substance:	2,5-diaminotoluene
Solvent:	DMSO
Batch:	/
Purity:	95%

Concentrations: 10^{-4}, 10^{-5}, 10^{-6} and 2 x 10^{-7} M
Treatment: 4 h treatment; fixation of the cells after overnight culture.
GLP: not in compliance

2,5-Diaminotoluene was investigated for the induction of unscheduled DNA synthesis (UDS) in primary hepatocytes isolated from rats and hamsters.
Cells were treated for 4 h with 2,5-diaminotoluene and (me-^{3}H)-thymidine (specific activity 70 -80 Ci/mmol) and further cultured overnight. Slides were then progressed for autoradiography.
Evaluation of autoradiography was done after 10 days exposure and methyl-green Pyronin Y staining. UDS was measured by counting the number of grains in each nucleus and subtracting the average number of grains present in 3 equal-sized adjacent cytoplasmic areas (net nuclear grain). The average net nuclear grain count for 60 cells per slide was calculated and the percentage of cells with > 5 net nuclear grains was determined.

Results
2,5-Diaminotoluene produced an increased average net nuclear grain count in the hepatocytes isolated from rat and hamster at the highest concentration tested compared to the untreated control cultures. Also the percentage of cells with > 5 net nuclear grains increased in a dose dependent manner.

Conclusion
Under the experimental conditions used 2,5-diaminotoluene induced unscheduled DNA synthesis and, consequently, is genotoxic in rats in this *in vitro* UDS test.

Ref.: 37

Comments
The present assay is reported in a paper from the open literature in which 7 azo dyes and their reduction products were tested in the *in vitro* unscheduled DNA synthesis test with hepatocytes isolated from rats and hamsters. Consequently, the raw data were not available. The results can only be used as supportive evidence.
The paper was published before the implementation of OECD guidelines.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline: OECD 474
Species/strain: Crl:NMRI BR
Group size: 5 mice/sex/group
Test substance: SAT 010935 (toluene-2,5-diamine sulfate)
Batch: 46847
Purity: 99.9%
Dose level: 0, 25, 50 and 90 mg/kg bw
Route: i.p.
Vehicle: ethanol/deionised water (20/80 v/v)
Sacrifice times: 24 h after treatment for all concentrations, 48 h for the control and mid dose
GLP: in compliance

Toluene-2,5-diamine sulfate was investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on the findings of two range finding studies to the highest tolerable dose. The LD50 was estimated to be about 123 mg/kg bw. 75% of this LD50 (90 mg/kg bw) was chosen to be the highest dose.
Therefore, in the main experiment mice were exposed to single i.p. doses of 0, 25, 50 and 90 mg/kg bw. Bone marrow cells were collected 24 h or 48 h (control, mid and high dose

only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE).
Bone marrow preparations were stained with a slightly modified Pappenheim method and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
In the high dose group 65% of the male and female mice died within 24 h after administration of toluene-2,5-diamine sulfate. From the "48 h sacrifice" group all animals died. The "48 h sacrifice" group was replaced by 5 male and 5 female mice treated with the mid dose. All other animals survived until the scheduled sacrifices.
In the high dose animals reduced motor activity and sedation was noted from toluene-2,5-diamine sulfate administration until premature death or sacrifice. In the mid dose animals reduced motor activity was noted on the day of toluene-2,5-diamine sulfate administration. No adverse effects were noted in the low dose group.
The amount of nucleated cells was slightly below the range of historical negative control data in the high dosed group and the mid dosed females of the "48 h sacrifice" group.
Treatment with toluene-2,5-diamine sulfate resulted in decreased PCE/NCE ratios compared to the untreated controls indicating that toluene-2,5-diamine sulfate had cytotoxic properties in the bone marrow.
Biologically relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found at any dose tested, neither 24 or 48 h after treatment and neither for male and females.

Conclusion
Under the experimental conditions used toluene-2,5-diamine sulfate did not induce micronuclei in bone marrow cells of treated mice and, consequently, toluene-2,5-diamine sulfate is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 38

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474
Species/strain:	NMRI
Group size:	5 mice/sex/group
Test substance:	A 5 (toluene-2,5-diamine sulfate)
Batch:	EFH 290394
Purity:	> 98%
Dose level:	0, 15, 50 and 150 mg/kg bw
Route:	orally
Vehicle:	PEG 400
Sacrifice times:	24 h after treatment for all concentrations, 48 h for the high dose only.
GLP:	in compliance

Toluene-2,5-diamine sulfate was investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on a preliminary study on acute toxic syndromes (like death, reduced spontaneous activity, eyelid closure, apathy) at various intervals of 1, 6, 24 and 48 h after start of treatment. In the main experiment mice were exposed to single oral doses of 0, 15, 50 and 150 mg/kg bw. Bone marrow cells were collected 24 h or 48 h (high dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE). Bone marrow preparations were stained with May-Grünwald/Giemsa and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results

In the preliminary study with oral exposure to 50 - 400 mg/kg bw toluene-2,5-diamine sulfate, death, reduction of spontaneous activity, eyelid closure and apathy were found. The seriousness of these clinical effects decreased with dose resulting in only eyelid closure in the 150 mg/kg bw group, the highest dose in the main experiment. At doses below 150 mg/kg bw, no clinical effects were observed.
Treatment with toluene-2,5-diamine sulfate did not result in decreased PCE/NCE ratios compared to the untreated controls indicating that toluene-2,5-diamine sulfate had no cytotoxic properties in the bone marrow. Therefore, sufficient exposure of bone marrow cells is questionable.
Biologically relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found at any dose tested, neither 24 nor 48 h after treatment.

Conclusion
Under the experimental conditions used toluene-2,5-diamine sulfate did not induce an increase in the number of micronucleated PCEs in bone marrow cells of treated mice and, consequently, toluene-2,5-diamine sulfate is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 39

Comment
Because the PCE/NCE ratio was not decreased, there are no indications for bone marrow cell exposure. In the preliminary study clinical effects indicated systemic availability of toluene-2,5-diamine sulfate. However, in the main experiment at the doses used these effects were not found. Therefore, this study is of limited value and can only be used as supportive evidence.

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*

Guideline:	OECD 486
Species/strain:	male Sprague Dawley rats
Group size:	3 rats per dose and sacrifice time
Test substance:	SAT 010935 (toluene-2,5-diamine sulfate)
Batch:	46847
Purity:	> 99.9%
Dose level:	0, 20, 40 and 80 mg/kg bw
Route:	oral gavage
Vehicle:	sterile water
Sacrifice times:	2 h and 14 h after dosing
GLP:	in compliance

Toluene-2,5-diamine sulfate was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Test concentrations were selected on the basis of information supplied by the sponsor. Clinical observations were carried out approximately 30 minutes after dosing and before sacrifice for the 14 h sampling time. Hepatocytes for UDS analysis were collected approximately 2 h and 14 h after administration of toluene-2,5-diamine sulfate. At least 90 minutes after plating the cells were incubated for 4 h with 10 µCi/ml ^{3}H-thymidine followed by overnight incubation with unlabelled thymidine. Evaluation of autoradiography was done after 14 days.
UDS was reported as net nuclear grain: the nuclear grain count subtracted with the average number of grains in 3 nuclear sized areas adjacent to each nucleus. The percentage of cells in repair (defined as cells with a net grain count of at least +5) was calculated for each animal. Unscheduled synthesis was determined in 50 randomly selected hepatocytes on 2 replicate slides per rat. Negative and positive controls were in accordance with the OECD guideline.

Results
Mortality was observed in animals dosed with 80 mg/kg bw for the 14 h sampling time. Therefore, slides prepared from animals treated with 80 mg/kg bw for both sampling times were not evaluated. Cytotoxic effects were not seen in rats treated with 20 and 40 mg/kg bw.
Neither a biologically relevant increase in mean net nuclear grain count nor in the percentage of cells in repair as compared to the untreated control was found in hepatocytes of any treated animal both for the 2 h and the 14 h treatment time. A positive (> 0) net nuclear grain count was found for one animal which was attributable to a cytotoxic effect of toluene-2,5-diamine sulfate as indicated by a reduced cytoplasm count.

Conclusion
Under the experimental conditions used toluene-2,5-diamine sulfate did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 40, 41

***In vivo* alkaline single cell gel electrophoresis (Comet) assay in mice and rats**

Guideline:	/
Species/strain:	ddY mice and Wistar rats
Group size:	4 male animals/group
Test substance:	2,5-diaminotoluene sulfate
Batch:	/
Purity:	> 98%
Dose level:	0 and 60 mg/kg bw
Route:	oral
Vehicle:	saline
Sacrifice times:	3, 8 and 24 h after start of treatment
Organs studied:	stomach, colon, liver, kidney, urinary bladder, lung, brain and bone marrow
GLP:	not in compliance

2,5-Diaminotoluene sulfate was investigated for the induction of DNA damage in the alkaline single cell gel electrophoresis (Comet) assay in various tissues of mice and rats. Test concentrations were based on the LD50 of acute toxicity experiments (0.5 x LD50). Mice and rats were orally exposed to 60 mg/kg bw 2,5-diaminotoluene sulfate and sacrificed 3, 8 and 24 h after treatment.
The animals were carefully observed for pharmacotoxic signs after treatment until sacrifice. Histopathological examination was conducted when positive results were obtained. Per organ 50 nuclei were examined for migration which was calculated as the difference between length of the whole comet and diameter of the head.

Results
An increase in DNA damage was exclusively found in the stomach of rats of the 8 h sampling time but not the shorter or longer sampling time. 2,5-Diaminotoluene sulfate did not induce a biologically relevant increase in DNA damage in any of the other tissues tested of the mice and rats.

Conclusion
Under the experimental conditions used 2,5-diaminotoluene sulfate induced significant DNA damage in the stomach of rats and, consequently, 2,5-diaminotoluene sulfate is genotoxic in the Comet assay with rats.

Ref.: 44

Comments

The present assay is reported in a paper from the open literature. The raw data were not available.
Effects observed only in the stomach may be due to localized irritation/toxicity. Since there was no information on histology provided in this study, the impact of localized irritation/toxicity can not be ruled out. The validity of this study, which was part of a large comparative investigation, has been questioned in the scientific community for several reasons. The study performance does not conform to the requirements that were recently published in order to improve the quality of comet assay data (Refs 42 and 43). Specifically, only one dose was evaluated, there is no historical control data to determine validity of each study and aid in interpretation of results, evaluation of only one slide and 50 nuclei per tissue and animal. For some substances, the positive results reported by Sasaki and colleagues could not be verified by others (Ref 43).
Therefore, the value of this single positive result at one sampling time in only one species is unclear.

Mouse spot test

Guideline:	/
Species/strain:	male T stock and female C57BL/6J mice
Group size:	4 mice per dose
Test substance:	2,5-diaminotoluene
Batch:	/
Purity:	/
Dose level:	0 and 30 mg/kg bw
Route:	i.p.
Vehicle:	HBSS
Scoring for mutations:	12 – 15 days after birth
GLP:	not in compliance

2,5-Diaminotoluene sulfate was investigated for the induction of somatic mutations in the mouse spot test. Female C57B1/6J mice were mated with C57B1/10J or T stock males. On day 10-14 of gestation, the female mice were treated *i.p.* with 30 mg/kg bw 2,5-diaminotoluene. At birth the number and morphology of the offspring were recorded. At 12 to 15 days after birth the offspring was scored for coat colour spots. Triethylenemelanine was used as positive control.

Results
There was no significant increase in recessive spots in the 2,5-diaminotoluene exposed pups observed after birth. Nor in the percentage "white midventral spots", which are thought to be related to toxicity of treatment.

Conclusion
Under the conditions of this test, 2,5-diaminotoluene did not induce somatic mutations in foetal cells following transplacental absorption and consequently, 2,5-diaminotoluene is not genotoxic (mutagenic) in mice.

Ref.: 46

Comment
The present assay is reported in a paper from the open literature. The experiment, performed before the development of the OECD guidelines, is a non-GLP study conducted with unspecified test material. It was not reported if the applied dose induced toxic effects in the treated females. Because the test does not comply with the requirements of the currently valid guideline, the result of this experiment can only be used as supportive evidence.

Mouse spot test

Guideline:	/
Species/strain:	male T stock and female C57BL/6 mice
Group size:	5 female mice per dose
Test substance:	Orex 111 (2,5-diaminotoluene dihydrochloride)
Batch:	/
Purity:	/
Dose level:	0, 15, 150 and 1500 mg/kg bw
Route:	dermal
Vehicle:	corn oil
Scoring for mutations:	days 12 and 24 after birth
GLP:	not in compliance

2,5-Diaminotoluene dihydrochloride was investigated for the induction of somatic mutations in the mouse spot test. Female C57B1/6J mice were mated with C57B1/10J or T stock males. On days 9, 10, and 11 post fertilisation 2,5-diaminotoluene dihydrochloride was administered dermally to a shaved patch on the dorsal side of the animal. At 12 and 24 days after birth the offspring was scored for coat colour spots. Benz(a)pyrene was used as positive control.

Results
No toxic effects in the treated animals and no effect on fertility or litter size were observed. There was no significant increase in treated animals with recessive spots as compared to the concurrent controls. 2,5-Diaminotoluene dihydrochloride exposure did not induce an increase in the percentage "white midventral spots" either, which are thought to be related to toxicity of treatment.

Conclusion
Under the conditions of this test, 2,5-diaminotoluene dihydrochloride did not induce somatic mutations in this mouse spot test and consequently, 2,5-diaminotoluene dihydrochloride is not genotoxic (mutagenic) in mice.

Ref.: 47

Comment
The experiment was not in compliance with GLP and was conducted before the development of the OECD guidelines. The test material is not specified. Because the test does not comply with the requirements of the currently valid guideline, the result of this experiment can only be used as supportive evidence.

Adapted from submission II, 2005
Two dominant lethal test have been conducted with toluene-2,5-diamine. Because these are non-GLP studies conducted with unspecified test material, only a brief description is provided. They are included since they add to the assessment of the genotoxicity of toluene-2,5-diamine *in vivo*. Though none of the tests fully comply with the requirements of the currently valid guidelines, taken together the results give further support for the conclusion that toluene-2,5-diamine is not mutagenic *in vivo*.

Dominant lethal test
Toluene-2,5-diamine dissolved in water was administered three times per week intraperitoneally at a dose of 20 mg/kg bw over 8 weeks to 20 male Charles-River rats. Each male was then housed with two females for 5 days. The females were killed 17 days later and the uteri were examined for live and dead foetuses, implantation and resorption sites. In total there were 460 live foetuses (12.4 per litter). Neither the percentages of litters with one or more resorptions, nor the number of mean resorptions per pregnancy or the percent resorptions per litter were different from the vehicle control values. Toluene-

2,5-diamine under the conditions of this test did not induce dominant lethal mutations in male rats. Under the conditions of this test toluene-2,5-diamine did not induce embryonic or foetal deaths by inducing chromosomal aberrations in germinal tissue. The test supports the conclusion that toluene-2,5-diamine sulfate is not mutagenic *in vivo* in germ cells.

Ref.: 48

Dominant lethal test

Toluene-2,5-diamine dihydrochloride in corn oil was applied topically to the shaved dorsal surface at doses of 1.5, 15, 150 mg/kg bw for 5 consecutive days to male mice. A weekly mating sequence with 2 females per week was started 2 days after the last application and was continued for 7 weeks. No attempt was made to prevent ingestion of the test substance during the treatment period. Fourteen days after the midweek of being caged with the male, the females were sacrificed. The final evaluation revealed no indication of dominant lethality. The positive control triethylenemelamine induced a significant dominant lethal response demonstrating the validity of the test system.

Ref.: 49

3.3.7. Carcinogenicity

Oral administration, mice

Guideline:	/
Species:	Mouse/B6C3F1
Group size:	50 animals per sex per dose level
Test substance:	toluene-2,5-diamine sulfate (CAS n° 6369-59-1)
Vehicle:	Diet (Wayne Lab-Blox® meal)
Batch:	Not indicated
Purity:	99% with 25 ppm iron, 0.6% volatiles, max. 0.1% moisture. No impurities were detected by thin-layer chromatography in two solvent systems
Dose level:	0.06, 0.1%
Route:	Oral, diet
Exposure period:	78 weeks, followed by an additional 16 – 19 weeks without treatment
GLP:	In compliance

Toluene-2,5-diamine sulfate was administered in the diet to groups of 50 mice per sex at either 0.06 or 0.2% for a period of 78 weeks followed by an additional 16 to 19 weeks of observation. These doses were selected after completion of a 4 week feeding study in male and female B6C3F1 mice. Because the test substance administration to the high and low dose groups was not begun simultaneously, each dosed group was assigned a separate control group of 50 animals per sex. Body weights were recorded twice weekly for the first 12 weeks and then at monthly intervals. Food consumption was monitored for seven consecutive days once a month for the first nine months and then for 3 consecutive days each month thereafter. Animals were monitored twice daily for mortality. A necropsy was performed on all animals that died, were sacrificed when moribund, or were sacrificed at study termination, and histopathological examinations were performed on major tissues, organs, and gross lesions. Slides were prepared from the following tissues: skin, subcutaneous tissue, lungs and bronchi, trachea, bone marrow, spleen, lymph nodes, thymus, heart, salivary gland, liver, gall bladder, pancreas, oesophagus, stomach, small intestine, large intestine, kidney, urinary bladder, pituitary, adrenal, thyroid, parathyroid, testis, prostate, seminal vesicle, brain, tunica vaginalis, muscle, ear, uterus, mammary gland, and ovary.

Results

Mean body weight was consistently depressed in high dose female mice compared to the corresponding control. There was no treatment-related effect on survival in either males or females.

A statistically significant increase in lung tumours (alveolar/bronchiolar adenomas or carcinomas) was observed in high dose female mice (high dose 8/45 (17%) vs. 1/45 (2%) in high dose control, P=0.016). The incidence was not significantly increased in low dose female mice (low dose 6/42 (14%) vs. 4/46 (9%) in low dose control). Results from high dose were not considered as convincing evidence of a treatment-related effect because the control for the high dose female mice were very low compared to the control for the low dose female mice (1/45 (2%) vs 4/46 (9%)), moreover no similar effects were observed in the male mice. The incidence of pituitary adenomas or carcinomas was significantly lower in high dose female mice (high dose 0/38 (0%) vs. 6/37 (16%) in high dose control, P=0.012). The incidence in low dose female mice was also lower, although not statistically significant (low dose 1/38 vs. 3/42 in low dose control).

Ref.: 51

Oral administration, rats

Guideline:	/
Species/Strain:	Rat/Fischer 344
Group size:	50 animals per sex per dose level except for high dose control groups which had 25 animals per sex
Test substance:	toluene-2,5-diamine sulfate (CAS n° 6369-59-1)
Vehicle:	Diet (Wayne Lab-Blox® meal)
Batch:	Not indicated
Purity:	99% with 25 ppm iron, 0.6% volatiles, max. 0.1% moisture. No impurities were detected by thin-layer chromatography in two solvent systems
Dose levels:	Low dose: 0.05% for 14 weeks, increased to 0.06% at week 15 (time weighted average = 0.06%) High dose: 0.2%
Route:	Oral, diet
Exposure period:	78 weeks, followed by an additional 28 to 31 weeks without treatment
GLP:	In compliance

Toluene-2,5-diamine sulfate was administered in the diet to groups of 50 rats per sex at either 0.05-0.06 or 0.2% for a period of 78 weeks followed by an additional 28 to 31 weeks of observation. These doses were selected after completion of a 4 week feeding study in male and female Fischer 344 rats. Because the test substance administration to the high and low dose groups was not begun simultaneously, each dosed group was assigned a separate control group of 50 animals per sex (low dose control) or 25 animals per sex (high dose control). Body weights were recorded twice weekly for the first 12 weeks and then at monthly intervals. Food consumption was monitored for seven consecutive days once a month for the first nine months and then for 3 consecutive days each month thereafter. Animals were monitored twice daily for mortality. A necropsy was performed on all animals that died, were sacrificed when moribund, or were sacrificed at study termination, and histopathological examinations were performed on major tissues, organs, and gross lesions. Slides were prepared from the following tissues: skin, subcutaneous tissue, lungs and bronchi, trachea, bone marrow, spleen, lymph nodes, thymus, heart, salivary gland, liver, pancreas, oesophagus, stomach, small intestine, large intestine, kidney, urinary bladder, pituitary, adrenal, thyroid, parathyroid, testis, prostate, seminal vesicle, brain, tunica vaginalis, muscle, ear, uterus, mammary gland, and ovary.

Results

Mean body weight was consistently depressed in high dose female rats. This trend was not as evident in the other groups of dosed rats. There was no treatment-related effect on survival in either males or females.

A statistically significant increase in the incidence of interstitial cell tumours of the testis was observed in male rats (low dose 43/48 (90%) vs. 33/45 (73%) in low dose control, P=0.039; high dose 47/48 (98%) vs. 19/24 (79%) in high dose control, P=0.014), but this was not considered treatment-related since the spontaneous incidence of these tumours in male rats is very high and variable. The incidence of pituitary adenomas in low dose male rats showed a statistically significant decrease relative to the corresponding control (low dose 3/45 (7%) vs. 12/41 (29%) in low dose control, P=0.006). A similar trend (not statistically significant) was seen in high dose male rats (high dose 3/40 (8%) vs. 3/21 (14%) in high dose control). The incidence of lung tumours (alveolar/bronchiolar adenomas or carcinomas) was significantly lower in high dose male rats (high dose 0/49 (0%) vs. 3/25 (12%) in high dose control, P=0.035), but this difference was not seen in low dose male rats (low dose 1/48 vs. 0/46 in low dose control). No significant increases in neoplasms were observed in female rats. The incidence of thyroid C-cell adenoma or carcinoma was significantly lower in high dose female rats (0/49 vs. 3/21 in high dose control), but the opposite trend (not statistically significant) was seen in low dose female rats (3/48 vs. 1/47 in low dose control).

Ref.: 51

Comment
The conclusion drawn in the NCI report for this study was: *"under the conditions of this bioassay, sufficient evidence was not provided to conclusively demonstrate the carcinogenicity of 2,5-toluenediamine sulphate in either Fischer 344 rats or B6C3F1 mice".*

Toluene-2,5-diamine in hair dye formulations together with hydrogen peroxide

Topical administration, mice

Guideline:	/
Species/strain:	Swiss-Webster mice
Group size:	28 Males and 28 females per treatment group and positive control, 14 males and 14 females in vehicle control group and 76 males and 17 females in untreated control group
Test substance:	toluene-2,5-diamine. Two hair dye formulations containing 3% toluene-2,5-diamine, 1.5% p-phenylenediamine and either 0.2% or 0.6% toluene-2,4-diamine. The dye preparation was mixed with equal volume 6% hydrogen peroxide before application.
Batch:	/
Purity:	not given
Dose:	0.05 ml of a solution containing toluene-2,5-diamine and hydrogen peroxide
Route:	Topical, 1 application weekly
Exposure:	2 years
GLP:	not in compliance

Two oxidation hair dye formulations containing 3% toluene-2,5-diamine, mixed with an equal volume of 6% hydrogen peroxide, were tested by topical application in groups of 28 male and 28 female mice weekly for 2 years. 7,12-dimethylbenz(a)anthracene (DMBA) (0.1%) was used as positive control (0.05 ml containing 2.5 and 10 µg DMBA). Each week they were weighed. When signs of marked irritation, ulceration, or tumour formation were evident, the application of the chemical was discontinued until the skin looked "normal". Tissue specimens were taken from all major organ systems and tumours of mice found dead during the study or sacrificed when moribund or at 2 year, the termination of the experiment. Body weight gains of mice in treated groups were not significantly different from those of mice in the untreated control groups. It was concluded that the male and female mice in all groups developed both malignant and benign neoplasms. There were no statistical difference between the sexes in the total number of neoplasms or in the incidence

of neoplasms of a particular organ and type. The incidence of skin neoplasms did not show statistically significant differences in any of the groups under test except for the positive control groups exposed to DMBA.

Ref.: 91

Guideline:	/
Species/strain:	Swiss-Webster mice
Group size:	50 Males and 50 females per treatment group and vehicle control
Test substance:	toluene-2,5-diamine. Two hair dye formulations containing 3.0% toluene-2,5-diamine, 1.5% p-phenylenediamine with either 0.2% toluene-2,4-diamine, 0.38% 2,4-diaminoanisole or 0.17% m-phenylenediamine prior to mixing with equal volume 6% hydrogen peroxide just prior to use.
Batch:	/
Purity:	not given
Dose:	0.05 ml of a solution containing toluene-2,5-diamine and hydrogen peroxide
Route:	Topical, 1 application weekly or 1 application every second week
Exposure:	18 months
GLP:	not in compliance

Two oxidation hair dye formulations containing 3% toluene-2,5-diamine, mixed with an equal volume of 6% hydrogen peroxide, were tested by topical application in groups of 100 mice weekly or once every two weeks for 18 months. 7,12-dimethylbenz(a)anthracene (DMBA) (0.1%) was used as positive control (0.05 ml containing 50 µg DMBA first 6 months, 10 µg next 4 months and 50 µg last 8 months). The mice were observed daily for signs of toxicity. Each week they were weighed, the skin graded for irritation and papillomas and other gross lesions were noted. Animals that died or that were killed because of general debility were autopsied and examined histopathologically when possible. At termination of the study, all survivors were weighed and killed and a gross autopsy was performed. There were no overt signs of systemic toxicity in any of the dye-treated groups. The survival varied from 58 to 80% except in the positive controls in which only 21% of the mice were alive after 18 months. Average body weights were comparable in all groups throughout the study. It is concluded that no evidence of carcinogenic activity was seen.

Ref.: 90

Topical administration, rats

Three experimental preparations in a carboxymethylcellulose gel were tested: formulation 1, containing 4% toluene-2,5-diamine (calculated as free base but used as sulfate): formulation 2, containing 3% toluene-2,5-diamine and 0.75% 2,4-diaminoanisole; and formulation 3, used as control without added dye intermediates. Each formulation was mixed with an equal volume of 6% hydrogen peroxide immediately before use, and 0.5 g of the mixture was applied to the dorsal skin. Three groups each of 50 male and 50 female Sprague-Dawley rats, 12 weeks old, were treated twice weekly for two years with either formulation 1 or 2 or left untreated. A fourth group of 25 male and 25 female rats were treated with formulation 3. No statistically significant differences were observed in tumour incidence between the experimental and control groups.

Ref.: 89

Comment
Hair dye formulations of toluene-2,5-diamine together with hydrogen peroxide have been tested in three experimental studies after topical application to mice or rats. The sensitivity of these studies may have been low as no response to hair dye formulations containing known carcinogens (toluene-2,4-diamine [EU carcinogenic. category 1B] or 2,4-

diaminoanisole [EU carcinogenic, category 1B] was observed. Thus, no conclusions with regard to carcinogenicity can be drawn from these studies.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

Guideline:	OECD 416
Species/strain:	Sprague-Dawley rats Him:OFA
Group size:	24 males and 24 females
Test substance:	p-toluenediamine
Batch:	präp. 139 (not characterised, see general comments)
Purity:	98.2%
Dose:	0, 5, 15 and 45 mg/kg bw/d in deionised water, 50 µl 25% ammonia per g test substance added
Route:	oral, gavage
Exposure:	once daily males: 70 d before mating females: prior to mating for 14 d, during mating period, gravidity, lactation until the end of the experiment F_1: from weaning for approximately 80 days until the end of the experiment
GLP:	in compliance

The test substance was administered to males for 70 days and to females for 14 days until mating. The animals were monogamously mated within the dose groups. Dams were further dosed until weaning of the pups. Starting on day 21 after birth the F_1 generation was dosed for approximately 80 d. After mating the F_2 generation was kept until weaning. The common parameters were evaluated (female sexual cycle, mating, insemination, gravidity, birth and litter data, postnatal weight and physiological development). Histopathology was performed for organs with obvious abnormalities, for parents without live offspring and for all parental animals of the control and the highest dose group. The reproduction organs (pituitary gland, mamma, vulva, vagina, cervix, uterus, tubes, ovaries, penis, testes, epididymides, ducti referentes, coagulation gland, prostate gland, vesicular gland) were examined microscopically.

Results
4 animals (one of P- and 3 of F_1-generation) died due to intubation-induced lesions. Body weight development, feed consumption and observation of the animals did not show substance treatment related differences. Observations and measurements in the pups of both generations until weaning did not show differences in the parameters evaluated. No detrimental effect on male and female fertility was found.

Conclusion
The NOAEL for reproductive toxicity was 45 mg/kg bw/d.

Ref.: 52

3.3.8.2. Teratogenicity

Study in rabbits

Guideline:	/
Species/strain:	New Zealand White Rabbit
Group size:	16 (vehicle control and dose groups), 18 (positive control Vitamin A in rape seed oil)
Test substance:	toluene-2,5-diamine sulfate in distilled water

Batch: 23005
Purity: /
Dose: 0, 10, 25, 50 mg/kg bw/d, positive control Vitamin A 6 mg/kg bw/d
Route: oral, gavage
Exposure: once daily from day 6 to 18 of gestation
GLP: /

The females were fertilised by natural mating. After coitus HCG was given i.v. to ensure ovulation. The animals were treated with 0, 10, 25, 50 mg/kg bw/d toluene-2,5-diamine sulfate in distilled water, the positive control group received 6 mg/kg bw/d Vitamin A from day 6 to 18 of gestation. The animals were examined daily for mortality and clinical signs. The body weights were determined on days 0, 6, 18 and 28 of gestation. On day 28 of gestation the animals were sacrificed, the foetuses were dissected and examined for congenital abnormalities and macroscopic changes. The common *sectio* parameters were recorded. Half of the foetuses were examined for skeletal and the other half for visceral abnormalities.

Results
1 female at 10 and 1 female at 25 mg/kg bw/d died; at 50 mg/kg bw, 3 females died presumably because of the intubation procedure. No clinical signs were observed. Body weights of the females in the dose groups were similar to the controls. The changes in the incidences of intrauterine death observed were not dose-related. The number and sex of the foetuses as well as the foetal body weights were not influenced by substance treatment. The frequencies of external abnormalities, visceral malformations and variations as well as skeletal defects exhibited no substance-related changes. The positive control (Vitamin A) did not show teratogenicity and only slight embryotoxicity.

Conclusion
The NOAEL of embryotoxicity and teratogenicity of toluene-2,5-diamine sulfate in rabbits is 50 mg/kg bw/d.

Ref.: 53

Study in rats

Guideline: /
Species/strain: Sprague Dawley rats
Group size: 23 mated females
Test substance: toluene-2,5-diamine sulfate in distilled water, Vitamin A in rape seed oil
Batch: 23005
Purity: /
Dose: 0, 10, 50 and 80 mg/kg bw/d, positive control Vitamin A 15 mg/kg bw/d
Route: oral, gavage
Exposure: once daily from day 6 to 15 of gestation
GLP: /

The test and control solutions were administered to the pregnant females from day 6 to 15 of gestation once daily by oral gavage. The animals were observed once daily for mortality and clinical signs. The body weights were determined on day 0, 6, 15 and 19 of gestation. On day 19 of gestation the animals were sacrificed by chloroform inhalation, the foetuses were dissected and examined for congenital abnormalities. The common *sectio* parameters were evaluated. Half of the foetuses were examined for skeletal and the other half for visceral abnormalities.

Results
2 animals (1 at 10 and 1 at 80 mg/kg bw/d) died possibly due to gavaging. No clinical signs were observed. The maternal body weights were markedly reduced at 80 and slightly

reduced at 50 mg/kg bw/d during the dosing period. For the period d 15-19 these changes were not observed. Post implantation loss was increased at 80 mg/kg bw/d. Number of foetuses, sex distribution and foetal weights were comparable to controls. Visceral and skeletal variations were in the normal range, no malformations were observed. The positive control showed a high incidence of skeletal malformations.

Conclusion
The NOAEL of toluene-2,5-diamine sulfate in rats for maternal toxicity is 50 mg/kg bw/d, the NOAEL of embryotoxicity and teratogenicity 80 mg/kg bw/d.

Ref.: 54

Comment
The use of a positive control is uncommon in teratogenicity studies.

3.3.9. Toxicokinetics

3.3.9.1. Toxicokinetics *in vitro*

Study 1, metabolism in primary hepatocytes of human, rat and mouse

Guideline: /
Cells: hepatocytes from humans (pooled from 3 male donors), male Sprague-Dawley rats and male ICR/CD-1 mice
Test substance: toluene-2,5-diamine sulfate
Batch: 2346
Purity: 98.3% (NMR)
Test concentration: 10 µM
Incubation time: 4 h
Study period: March 2003
GLP: in compliance

Phase I and II metabolism of the test substance was examined *in vitro* using comparatively primary human, rat and mouse hepatocytes. Cryopreserved primary human hepatocytes that were phenotyped by the supplier regarding NAT2 activities were pooled in this study to achieve average activities; in summary, a rapid N-acetylation phenotype was expected. SD-rats are rapid metabolizers while CD-1 mice were reported to be a mixed population of rapid and slow metabolizers. The cell viability appeared to be unaffected over the incubation period of 4 h. The functionality of the system was tested using marker reactions for CYP2A6, CYP1A, CYP2A and CYP2B. Additional marker reactions for CYP1A1/2, CYP2E1 and human NAT1/NAT2 were included.

Results
Toluene-2,5-diamine sulfate was extensively metabolized by all hepatocytes (order rat ≈ mouse > human). With human hepatocytes the substance was completely metabolized after 4 h. For all three species N-acetylation was the major metabolic reaction and the results indicate that toluene-2,5-diamine sulfate is substrate for both types on N-acetyltransferases NAT1 and NAT2. Only the mono-N-acetylated metabolite was detected under the conditions of the study. Mouse hepatocytes additionally showed extensive hydroxylation of toluene-2,5-diamine sulfate.

Ref.: 56

Comment
No firm conclusions can be drawn from the study results with regards to the relative activity or preference of human NAT1 and NAT2 towards the substrate toluene-2,5-diamine.

Study 2, human hepatic metabolism *in vitro*

Guideline:	/
Cells:	hepatocytes from humans (pooled from 4 female donors) pooled human liver microsomes bacterially expressed human CYP isozymes CYP1A1, CYP1A2, CYP1B1, CYP2C9, CYP2C19, CYP2D6, CYP3A4
Test substance:	[ring-U-^{14}C]-toluene-2,5-diamine sulfate
Batch:	CFQ13783, batch 1 [ring-U-^{14}C]-toluene-2,5-diamine sulfate Lot 16825DR Sigma Aldrich non-labelled toluene-2,5-diamine sulfate
Purity:	radiochemical purity 98.2% (HPLC) non-labelled toluene-2,5-diamine sulfate 98.3% (NMR)
Test concentration:	10 µM and 100 µM
Incubation time:	hepatocytes 4 h hepatic microsomes 20 min recombinant human isozymes 60 min
Study conduct:	2004
GLP:	/

The human hepatic metabolism *in vitro* of [ring-U-^{14}C]-toluene-2,5-diamine sulfate was investigated in hepatocytes from humans (pooled from 4 female donors, each phenotyped with regards to the isoenzymes investigated), pooled human liver microsomes and bacterially expressed human CYP isozymes CYP1A1, CYP1A2, CYP1B1, CYP2C9, CYP2C19, CYP2D6, CYP3A4. The formation of metabolites was proven by LC/MS.

Results
With human microsomes (10 µM and 100 µM toluene-2,5-diamine sulfate) there was no evidence for the production of oxidative metabolites while the positive control substance 2-aminofluorene yielded a number of oxygenated metabolites. When toluene-2,5-diamine sulfate (10 µM and 100 µM) was incubated with recombinant CYP isozymes no metabolites were detected while the positive control substance 2-aminofluorene yielded a isozyme-specific pattern of oxidative metabolites. When toluene-2,5-diamine sulfate (10 µM and 100 µM) was incubated with human hepatocytes an indication was found by mass spectrometry (MS) for the formation of a mono-acetylated derivative. No glucuronides, sulphate esters or mono-hydroxylated metabolites were detected.

Ref.: 57

3.3.9.2. Toxicokinetics *in vivo*

Study 1, absorption, distribution, metabolism and excretion in Kyoto rats after a single oral or dermal dose

Guideline:	OECD 417 (1984) OECD 427 (draft 2000)
Species/strain:	Wistar Kyoto rats, WKY/NR Crl BR (inbred)
group size:	4 females in the mass balance groups 6 females in the toxicokinetics groups
Test substance:	toluene-2,5-diamine sulfate oral: in water at pH 7 dermal: in water:acetone 1:1 at pH 7
Batch:	3362-259 [ring-U-^{14}C]-toluene-2,5-diamine sulfate CFQ13783, batch 1 [ring-U-^{14}C]- toluene-2,5-diamine sulfate 2346 (non-labelled toluene-2,5-diamine sulfate)
Purity:	99.3% (HPLC) (radiochemical purity) 98.3% (NMR) (non-labelled toluene-2,5-diamine sulfate)
Dose levels:	oral: 2.5, 25 mg/kg bw

	dermal: 0.5 mg/cm^2 equal to 33.3 mg/kg bw
Route:	oral, gavage or dermal (30 min)
Study conducted:	2004
GLP:	in compliance

Rats were dosed orally with 2.5 or 25 mg/kg bw and dermally with 33.3 mg/kg bw (corresponding to 0.5 mg/cm^2). In the oral mass balance study urine and faeces were collected for 0-8, 8-24, 24-48, 48-72, 72-96 h intervals and several tissues were collected. In the dermally dosed group urine and faeces were collected at 24 h intervals and animals were sacrificed at 120 h. In the toxicokinetic groups, blood was sampled at the time points 0.25, 0.5, 1, 2, 4, 6, 24, 48 and 72 h after dosing.

Results
The mean cumulative recovery in the urine after 96 h was 62.2 (low dose) and 72.9% (high dose) while the figures for the faeces were 31.4% and 22.0%, respectively. The mean mass balance (oral) was ca. 98%. The average dermal absorption was 16% (= 0.101 mg/cm^2) but together with the amount remaining in the skin the absorbed amount was calculated as 20% of the applied dose (= 0.126 mg/cm^2). The mean mass balance (dermal) was nearly 100%. After oral dosing three metabolites were observed in the urine, two of them probably mono-N-acetylated. The largest peak was identified as N,N'-diacetyl-toluene-2,5-diamine which was also found in faeces. The metabolite pattern after dermal dosing was similar to that after oral dosing.
Following oral dosing of 2.5 and 25 mg/kg bw toluene-2,5-diamine the AUC values were 17.59 and 174 mg_{eq} x h/l, respectively. The dermal dose of 33.3 mg/kg bw (equal to 0.5 mg/cm^2) resulted in an AUC of 4.39 mg_{eq} x h/l.

Ref.: 58

Comment
The Kyoto strain was used because it is a slow acetylator phenotype.

Study 2, absorption, distribution, metabolism and excretion in Kyoto rats after a single intravenous dose

Guideline:	OECD 417 (1984)
Species/strain:	Wistar Kyoto rats, WKY/NR Crl BR (inbred)
Group size:	4 females (mass balance), 6 females (kinetics)
Test substance:	toluene-2,5-diamine sulfate in water at pH 7
Batch:	CFQ13783, batch 1 [ring-U-^{14}C]-toluene-2,5-diamine sulfate
	2346 (non-labelled toluene-2,5-diamine sulfate)
Purity:	98.2% (HPLC) (radiochemical purity)
	98.3% (NMR) (non-labelled toluene-2,5-diamine sulfate)
Dose levels:	2.5 mg/kg bw
Route:	intravenous, single application
Study conducted:	2004
GLP:	in compliance

2 groups were used, one (n=4) for mass balance and one (n=6) for toxicokinetics. The animals were dosed intravenously with 2.5 mg/kg bw [ring-U-^{14}C]-toluene-2,5-diamine sulfate. In the mass balance groups urine and faeces were collected for 0-8, 8-24, 24-48, 48-72, 72-96 h intervals and several tissues were collected. In the toxicokinetic groups blood was sampled at the time points 5, 15, and 30 min and 1, 2, 4, 6, 24, and 48 h after dosing.

Results
Urinary excretion accounted for 54% and excretion via faeces for 27% of the applied dose. 81 - 87% of the administered dose was excreted during the study period while 4.2 - 9.5%

remained in the carcass. In urine 2 major metabolites were observed and the largest peak was identified as N,N-diacetyl-toluene-2,5-diamine which was also found in faeces. The metabolite pattern was similar to that after oral administration (see Ref. 58).

Ref.: 59

Comment
The bioavailability in Kyoto rats (derived from comparison to iv administration) after oral dosing was > 90% while 2% bioavailability was found after dermal application.

Study 3, pharmacokinetics in Sprague-Dawley rats after a single oral or dermal dose

Guideline:	OECD 417 (1984) OECD 427 (draft 2000)
Species/strain:	Sprague-Dawley rats, Crl:CD (outbred)
Group size:	6 females
Test substance:	toluene-2,5-diamine sulfate oral groups 1 and 2: in water at pH 7 dermal group 3: in water:acetone 1:1 at pH 7 dermal group 4: vehicle 81905108B
Batch:	3362-259 [ring-U-^{14}C]-toluene-2,5-diamine sulfate CFQ13783, batch 1 [ring-U-^{14}C]-toluene-2,5-diamine sulfate 2346 (non-labelled toluene-2,5-diamine sulfate)
Purity:	99.3% (HPLC) (radiochemical purity) 98.3% (NMR) (non-labelled toluene-2,5-diamine sulfate)
Dose levels:	oral: 2.5, 25 mg/kg bw dermal: 0.5 mg/cm^2 equal to 33.3 mg/kg bw
Route:	oral, gavage or dermal (30 min)
Study conducted:	2004
GLP:	in compliance

Rats were dosed orally with 2.5 or 25 mg/kg bw and dermally with 33.3 mg/kg bw (corresponding to 0.5 mg/cm^2). Blood was sampled at the time points 0.25, 0.5, 1, 2, 4, 6, 24, 48 and 72 h after dosing.

Results
Oral absorption of toluene-2,5-diamine sulfate was rapid with T_{max} values of 1 h. AUCs were 8.53 (low dose) and 112 mg_{eq} x h/l (high dose). After dermal application T_{max} was 2 h and the AUCs were 5 (vehicle water/acetone) and 2.27 mg_{eq} x h /l (vehicle formulation).

Ref.: 60

Study 4, toxicokinetics in Sprague-Dawley rats after a single intravenous dose

Guideline:	OECD 417 (1984)
Species/strain:	Sprague-Dawley rats, Crl:CD (outbred)
Group size:	6 females
Test substance:	toluene-2,5-diamine sulfate in water at pH 7
Batch:	CFQ13783, batch 1 [ring-U-^{14}C]-toluene-2,5-diamine sulfate 2346 (non-labelled toluene-2,5-diamine sulfate)
Purity:	98.2% (HPLC) (radiochemical purity) 98.3% (NMR) (non-labelled toluene-2,5-diamine sulfate)
Dose levels:	2.5 mg/kg bw
Route:	intravenous, single application
Study conducted:	2004
GLP:	in compliance

A single intravenous dose (2.5 mg/kg bw) of [ring-U-^{14}C]-toluene-2,5-diamine sulfate was administered to 6 animals. Blood was sampled at the time points 5, 15, and 30 min and 1, 2, 4, 6, 24, and 48 h after dosing.

Results
The radioactivity in plasma was eliminated with a half-life of 28.3 h.

Ref.: 61

Comment
The bioavailability in Sprague-Dawley rats (derived from comparison to i.v. administration) after oral dosing was 69% while 2% bioavailability was found after dermal administration in a formulation.

Study 5, absorption, disposition and elimination in humans following application to scalp

Guideline:	/
Species:	human
Group size:	5 adult males per formulation
Test substance:	[^{14}C-CH_3]-toluene-2,5-diamine sulfate in a hair dye formulation
Batch:	/
Purity:	/
Radioactivity:	19.98 µCi [^{14}C-CH_3]-toluene-2,5-diamine sulfate diluted with 1.65 g non-labelled toluene-2,5-diamine sulfate in 100 g hair dye formulation
Treatment:	A total of 45 g of a formulation containing 0.825% resorcinol and 0.825% [^{14}C-CH_3]-toluene-2,5-diamine sulfate mixed with water (formulation I) or 6% hydrogen peroxide (formulation II) corresponding to 180 mg/cm^2 formulation (1.48 mg/cm^2 [^{14}C-CH_3]-toluene-2,5-diamine sulfate, 17.98 nCi/cm^2)
Exposed area:	250 cm²
Route:	dermal on scalp for 30 minutes
Year of report:	1981
GLP:	/

The absorption and elimination of [^{14}C-CH_3]-toluene-2,5-diamine sulfate following topical application of 2 formulations to hair-bearing scalp was studied using 5 human male adults per group. 45 g of a formulation containing 0.825% resorcinol and 0.825% [^{14}C-CH_3]-toluene-2,5-diamine sulfate mixed with water (formulation I, non-oxidative conditions) or 6% hydrogen peroxide (formulation II, oxidative conditions) corresponding to 1.48 mg/cm^2 [^{14}C-CH_3]-toluene-2,5-diamine sulfate was applied on the scalp for 30 minutes. The formulation was rinsed off and the hair was shampooed. Radioactivity in urine and faeces as well as in in blood samples taken at certain time periods was measured.

Results
Following application of formulation I (non-oxidative conditions), the sum of excreted radioactivity in urine (0-48 h) was 2.31% (8.55 mg of 370 mg applied) whereas the respective figure for formulation II (oxidative conditions) was 0.78% (2.89 mg of 370 mg applied). The mean total elimination rate of radioactivity in urine and faeces was 4.81 ± 0.62% (formulation I, non-oxidative conditions) and 1.31 ± 0.14% (formulation II, oxidative conditions) of the applied dose. The absorption rates of toluene-2,5-diamine sulfate were 71 ± 9.26 $\mu g_{eq}/cm^2$ (formulation I, non-oxidative conditions) and 20 ± 2.02 $\mu g_{eq}/cm^2$ (formulation II, oxidative conditions).

Half life in blood ranged from 1.2 – 2.7 h and AUCs were calculated to be 41.6 ± 1.7 ng_{eq} x h/ml (formulation I, non-oxidative conditions) and 9.2 ± 3.1 ng_{eq} x h/ml (formulation II, oxidative conditions).

Ref.: 62

Comment of the SCCS
This study was performed using only 5 persons per formulation and a concentration of 0.825% on the scalp. In contrast, the applicant's dossier intends the use of an on-head concentration of 3.6% toluene-2,5-diamine sulfate. The mean recovery was only 86 and 47% under non-oxidative and oxidative conditions, respectively. Study reporting was not according to modern standards.

Study 6, *In Vivo* Toxicokinetic Study with Dermal Application in the Sprague Dawley rat (Submission III, 2010)

Guideline:	OECD 417/427 (1984/2004)
Species/strain:	Rat, strain Sprague Dawley, Crl;CD (outbred, SPF quality), females
Group size:	4 animals per dose group
Test substance:	toluene-2,5-diamine sulfate
Batch:	CFQ40199 batch 1 [ring-^{14}C(U)]-toluene-2,5-diamine sulfate 2346 (non-radiolabelled toluene-2,5-diamine sulfate)
Purity:	Radiochemical purity: 99.0% by HPLC - [ring-^{14}C(U)]-toluene-2,5-diamine sulfate, Batch CFQ40199, batch 1 Non-radiolabelled toluene 2,5-diamine sulfate: >99% at 254 nm
Dose levels (actual):	2.1 mg/mL; 21 µg/cm^2 - dosing surface area 20 cm^2; 1.4 mg/kg bw 7.7 mg/mL; 75 µg/cm^2 - dosing surface area 20 cm^2; 6.0 mg/kg bw 24 mg/mL; 224 µg/cm^2 - dosing surface area 20 cm^2; 19.5 mg/kg bw 72 mg/mL; 734 µg/cm^2 - dosing surface area 20 cm^2; 63.2 mg/kg bw 7.3 mg/mL; 80 µg/cm^2 - dosing surface area 5 cm^2; 1.5 mg/kg bw 72 mg/mL; 677 µg/cm^2 - dosing surface area 5 cm^2; 13.3 mg/kg bw
Route:	Dermal
Dose volume:	0.2 ml for 20 cm^2 surface area; 0.05 ml for 5 cm^2 surface area
Vehicle:	Ammonia (25%)/Milli-Q water/hair dye formulation 81905108B (5/15/80); final concentration of ammonia was 1%
Dosing Schedule:	Single application for 30 minutes
Study date:	7/2008 – 8/2008
GLP:	In compliance

A single dose of [^{14}C]-toluene-2,5-diamine sulfate was administered by dermal application to groups of 4 female Sprague Dawley rats. Approximately 24 h prior to treatment, the fur was shaved on the back of the animals. For dose groups involving a total dosing surface area of 20 cm^2, two rubber "O"-rings with an internal surface area of 10 cm^2 were glued to the skin with Histoacryl Tissue Adhesive (B. Braun, Meslungen, Germany). For dose groups involving a total dosing surface area of 5 cm^2, one rubber "O"-ring with an internal surface area of 5 cm^2 was glued onto the skin. Animals wore a collar to prevent grooming of the treated area. Exposure was terminated 30 minutes post-application by cleaning the application site with cotton swabs moistened with a 10% aqueous shampoo solution or water.

Blood samples (ca. 200 µl) were collected from the tail vein at 0.5, 1, 2, 3, 4, 8, 24, and 48 h after application. Within a maximum of 1 hour after sampling, blood was centrifuged to obtain plasma, which was stored at ≤ -75 ºC until determination of radioactivity. After the final blood sample was collected, the animals were euthanized by an O_2/CO_2 procedure. The total amount of radioactivity in plasma samples was measured by liquid scintillation counting.

Concentrations of radioactivity in plasma were calculated as mg equivalents of toluene-2,5-diamine sulfate/kg sample based on the specific activity of the radiolabeled toluene-2,5-diamine sulfate formulation. Toxicokinetic parameters were calculated from the curves constructed from the individual values at each time point using the WinNonlin 5.2 program. Parameters that were calculated included C_{max}, t_{max}, t_{last}, AUC_{last}, AUC_{∞}, λ_z, and $t_{1/2}$. C_{max} and AUC values were also dose-normalized to 1 mg/kg bw.

Results and Discussion

Dermal absorption was rapid with t_{max} reached at 0.5 - 1 h after dosing in all groups. C_{max} values of the groups 1 – 4 ranged from 0.06 ± 0.06 mg/kg plasma (group 1, 1.4 mg/kg bw) to 6.5 ± 7.0 mg/kg plasma (group 4, 63.2 mg/kg bw). Plasma AUC values for each of the dose groups are summarized in the table below. For Groups 1, 5 and 6 values for plasma $AUC_{(0-\infty)}$ were only approximations because of the lack of measurable plasma concentration values in the terminal elimination phase of the plasma concentration *versus* time profile.

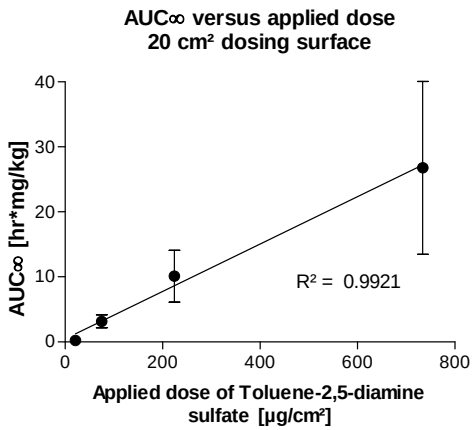
SUMMARY TABLE

Group	Dose Level	Dosing area (cm^2)	Dose level ($\mu g/cm^2$)	AUC_{∞} (hr*mg/kg)[1]	#AUC_{∞} (hr*mg/kg/(mg/kg))[1]	#AUC_{last} (hr*mg/kg/(mg/kg))[1]
1	1.4 mg/kg; 2.1 mg/mL	20	21	0.232* ± 0.184	0.153* ± 0.109	0.129 ± 0.104 (t_{last}: 4-8 h)
2	6.0 mg/kg; 7.7 mg/mL	20	75	3.16 ± 2.00	0.536 ± 0.369	0.508 ± 0.359 (t_{last}: 24 h)
3	19.5 mg/kg; 24 mg/mL	20	224	10.1 ± 7.94	0.513 ± 0.395	0.472 ± 0.399 (t_{last}: 3-24 h)
4	63.2 mg/kg; 72 mg/mL	20	734	26.8 ± 26.6	0.431 ± 0.427	0.411 ± 0.426 (t_{last}: 8-24 h)
5	1.5 mg/kg; 7.3 mg/mL	5	80	0.494* ± 0.393	0.327* ± 0.247	0.258 ± 0.244 (t_{last}: 8 h)
6	13.3 mg/kg; 72 mg/mL	5	677	2.05* ± 0.652	0.156* ± 0.0531	0.130 ± 0.0467 (t_{last}: 8 h)

* = approximation

[1] Values represent mean ± SD

Analysis of the dose groups 1 - 4 involving a 20 cm^2 dosing surface indicates that plasma $AUC_{(0-\infty)}$ increased proportionately with increasing applied dose (expressed as $\mu g/cm^2$). A linear regression analysis gave a correlation coefficient of 0.9921 (see figure below).



Plasma AUC values were greater after application to skin surface area of 20 cm^2 *versus* 5 cm^2 when comparing approximately equally applied doses (Groups 2 and 5 with applied doses of 75 and 80 $\mu g/cm^2$, respectively, and Groups 4 and 6 with applied doses of 677 and

734 µg/cm^2, respectively). However, in Groups 5 and 6 (5 cm^2 surface area of application) values for plasma $AUC_{(0-\infty)}$ were only approximations because of the lack of measurable plasma concentration values in the terminal elimination phase of the plasma concentration *versus* time profile. Therefore, a quantitative comparison of mean $AUC_{(0-\infty)}$ values for different skin surface areas with similar µg/cm^2 doses was not considered appropriate.

Ref.: 12 (subm III)

Study 7, toxicokinetics of [^{14}C] toluene-2,5-diamine sulfate following a single oral administration to Sprague-Dawley rats (Submission III, 2010)

Guideline:	OECD 417
Species/strain:	Sprague-Dawley rat
Group size:	6 per sex per group
Test substance:	toluene-2,5-diamine sulfate (non-radiolabelled) [Ring-^{14}C(U)]-toluene-2,5-diamine sulfate, specific activity 75 mCi/mmol
Batch:	CFQ40808, Batch B1- [ring-^{14}C(U)]-toluene-2,5-diamine sulfate R0022279 – (Non-radiolabelled toluene-2,5-diamine sulfate)
Purity:	Radiochemical purity: 98.9% by HPLC [ring-^{14}C(U)]-toluene-2,5-diamine sulfate Non-radiolabelled toluene-2,5-diamine sulfate: 99.5% by NMR
Vehicle:	Reverse osmosis deionized water, pH 5.0 $\pm$ 0.3
Dose levels:	5 and 10 mg/kg bw (containing approximately 100 µCi of radioactivity per dose)
Route:	Oral gavage
Dosing:	Single administration
Study date:	04/2010 – 09/2010 (performed outside the EU)
GLP:	In compliance

A single dose of [^{14}C]-toluene-2,5-diamine sulfate was administered orally by gavage at 5 or 10 mg/kg bw to fasted animals in a dose volume of 10 ml/kg bw. Dosing solutions were prepared on the day of dosing using the same vehicle used for the 91-day oral toxicity study (Charles River study VK00169, 18 August 2010). Blood (*circa* 0.3 ml) was collected via the cannulated jugular or carotid artery at 0.25, 0.5, 1, 2, 4, 8, 24, 48, and 72 h post dose and transferred to tubes containing K_2-EDTA anticoagulant and kept on wet ice until centrifugation. Within 1 hour of collection blood was centrifuged refrigerated (4 ºC) at 2700 rpm for approximately 10 min to separate plasma. Blood was also collected via the cannulated jugular or carotid from undosed control animals (Group 1, one male and one female). The plasma obtained from these animals was used to determine background levels of radioactivity. Duplicate weighed aliquots of dose formulation and plasma samples were mixed directly with liquid scintillation fluid for radioactivity measurement.

Plasma concentrations of radioactivity in dpm/g and mass eq/g were calculated based on the measured specific activity of the radiolabelled test material in the dose formulation. The toxicokinetic profile for each animal was characterized by non-compartmental analysis of the plasma radioactivity concentration data using validated computer software. The area under the radioactivity plasma concentration *versus* time curve (AUC) was calculated using the linear trapezoidal method. The parameters calculated included the following: C_{max}, T_{max}, $AUC_{(0\text{-tlast})}$, $T_{1/2}$, $AUC_{(0-\infty)}$.

Results and Discussion

Toxicokinetic parameters calculated from the concentration *versus* time profiles for each treatment group (5 and 10 mg/kg bw) are presented below:

Pharmaco-kinetic Parameter	Units	Group 2 (5 mg/kg bw) (mean[a] ± SD)			Group 3 (10 mg/kg bw) (mean[a] ± SD)		
		M	F	M and F	M	F	M and F
T_{max}	H	0.40	0.57	0.567	0.6	0.5	0.534
C_{max}	μg_{eq} / ml	6.42 ± 0.471	7.24 ± 0.446	6.83 ± 0.613	12.6 ± 1.11	13.3 ± 0.984	12.9 ± 1.07
$AUC_{(0\text{-}tlast)}$	μg_{eq} x h/ml	19.2 ± 2.68	25.1 ± 4.53	22.1 ± 4.72	40.7 ± 3.25	51.2 ± 4.04	46.0 ± 6.53
$t_{1/2}$	h	12.8 ± 6.14	12.6 ± 4.28	12.7 ± 5.05	14.2 ± 4.37	11.3 ± 5.94	12.8 ± 5.20
$AUC_{(0\text{-}\infty)}$	μg_{eq} x h/ml	19.3 ± 2.65	25.3 ± 4.51	22.3 ± 4.72	41.0 ± 3.30	51.6 ± 3.92	46.3 ± 6.51

[a] Median value reported for T_{max}

The highest mean observed concentration (C_{max}) was similar for both males and females and increased proportionally with an increase in dose. The C_{max} was observed between 0.40 and 0.60 h post dose, suggesting a rapid absorption of the test material following the oral dose. Following the C_{max}, the plasma radioactivity concentrations declined but were still detectable at 72 h post dose for both groups. Systemic exposure based on $AUC_{(0\text{-}\infty)}$ was slightly higher for females as compared to males and increased proportionally with an increase in dose. The mean terminal elimination half life ($T_{1/2}$) was similar for both sexes and ranged from 11.3 to 14.2 h.

Ref.: 14 (subm III)

Exposure to 2 commercial hair dye products (brown-red colour shade and black-brown colour shade) was investigated in 2 females in Germany (Schettgen *et al.*, 2011). Both were genotyped as slow acetylators (NAT2). Urinary samples were collected for a time period of 48 h after personal application of the hair dye creams which were labelled to contain toluene-2,5-diamine. The concentrations of the indicated ingredient were unknown. The urine samples were heated with 37% hydrochloric acid for 1 h at 80 °C in order to hydrolyse the amine conjugates and then analysed for aromatic diamines as well as for o-toluidine and 4-aminobiphenyl (as possible contaminants) using a GC-MS method. The cumulative excreted amounts of toluene-2,5-diamine were 700 µg and 1500 µg, respectively. The kinetics of urinary toluene-2,5-diamine after dermal application showed a phase of absorption and distribution within the body of about 12 h. Excretion mainly followed a first-order kinetics with a urinary half-life of about 8 hours.

Ref.:AR3

Study 8, consumer exposure to an oxidative hair dye. A [^{14}C]-labelled toluene-2,5-diamine mass balance study (Submission IV, 2012)

Guideline:	/
Species:	Human
Group Size:	16 Healthy adult male and female subjects per group
Methodology:	Open study with a single application of an oxidative hair dye mixture onto the hair
Test Substance:	2,5-Diamino[ring-U-^{14}C]-toluene sulfate - 80 mCi/mmol; 2.96 GBq/mmol; radiochemical purity 97.8% (HPLC)
Batch:	CFQ41174
Test Formulations:	Isotopic dilution of 2,5-Diamino[ring-U-14C]-toluene sulfate in a dark shade oxidative hair dye formulation with a final on-head concentration of either 1.5% or 4.0% toluene-2,5-diamine (expressed as free base)

	plus couplers, following mixing with hydrogen peroxide-containing developer (1:1, w/w).
Batch:	DTF 0938002 BF0 2 (1.5% toluene-2,5-diamine formulation); DTF 0938002 AF0 3 (4% toluene-2,5-diamine formulation)
Study period:	Aug-Sep 2011
GCP Status:	In compliance; ICH Guideline for Good Clinical Practice (ICH topic E6, adopted 01-05 1996 and implemented 17-01 1997)

In this study, two groups of human subjects (N=16 per group, 27 males and 5 females, age range 18-45 years) received a single application of an oxidative hair dye mixture by professional hairdressers. The final on-head concentration of [^{14}C]-toluene-2,5-diamine in the hair dye mixture applied was either 1.5% or 4.0% (expressed as free base). Both of the oxidative hair dye mixtures also contained m-aminophenol (A015), 4-amino-2-hydroxytoluene (A027), and 2,4-diaminophenoxyethanol dihydrochloride (A042) as couplers. The duration of the hair colouring exposure was 30 minutes. Following hair colouring, the dye was rinsed off, and the hair was shampooed, dried and clipped. Urine and faeces were collected quantitatively for 48 hours, and blood samples were taken prior to hair dyeing and at 2, 4, 6, 8, 10, 24, and 48 hours after hair colouring was begun. A protective cap was worn after clipping of the hair until the scalp was washed the following morning in order to collect radiolabelled substance exfoliated from the scalp during that time. Urine and faeces samples, as well as clipped hair, protective caps, wash water, and other materials (combs, brushes, towels, and gloves) were analysed for [^{14}C] radioactivity by liquid scintillation counting to calculate an overall mass balance. Plasma samples were analysed for [^{14}C] radioactivity to evaluate plasma kinetics of [^{14}C]-toluene-2,5-diamine equivalents. Calculated plasma kinetic parameters included T_{max}, C_{max}, $AUC_{(0\text{-}48hr)}$, $AUC_{(0\text{-}\infty)}$, and $T_{1/2}$.

Results

The overall mean mass balance obtained in this study was 98.85 $\pm$ 2.70% and 98.49 $\pm$ 2.97% for the low (1.5%) and high (4.0%) concentrations, respectively. The bulk of radioactivity was recovered in washing water and coloured hair. The washing water contained a mean of 56.28 $\pm$ 6.34% and 66.48 $\pm$ 5.40% of the applied radioactivity for the low and high concentration mixtures, respectively. The coloured hair contained a mean of 41.55 $\pm$ 6.09% and 31.16 $\pm$ 5.18% of the applied radioactivity for the low and high concentration mixtures, respectively.

Mean urinary excretion represented 0.83 $\pm$ 0.26% and 0.83 $\pm$ 0.42% of the applied radioactivity while mean faecal excretion represented 0.035 $\pm$ 0.02% and 0.042 $\pm$ 0.04% for the low and high concentration mixtures, respectively. Taken together, excretion in the 48 hour urine and faeces accounted for 0.86 $\pm$ 0.27% and 0.87 $\pm$ 0.45% of the applied radioactivity which would correspond to 6.28 $\pm$ 1.72 mg and 17.99 $\pm$ 8.76 mg toluene-2,5-diamine equivalents for the low and high concentration hair dye mixtures, respectively.

Plasma kinetic results are summarised in the following table.

Parameter	Units	Mean	SD	Min	Max
		1.5% toluene-2,5-diamine			
T_{max}	hr	2.4	0.8	2.0	4.0
C_{max}	ng eq/mL	99.1	38.4	46	180
$AUC_{(0-48h)}$	ng eq*hr/mL	1189	390	543	2128
$AUC_{(0-\infty)}$	ng eq*hr/mL	1241	402	583	2175
$T_{1/2}$	hr	10.1	1.3	8.5	12.4
Excretion $urine_{(0-48h)}$	% of dose	0.826	0.25	0.40	1.31
Excretion $faeces_{(0-48h)}$	% of dose	0.035	0.024	0.005	0.096
Excretion $total_{(0-48h)}$	% of dose	0.86	0.27	0.41	1.41
		4.0% toluene-2,5-diamine			
T_{max}	hr	2.6	1.0	2.0	4.0
C_{max}	ng eq/mL	266	145	111	599
$AUC_{(0-48h)}$	ng eq-hr/mL	3341	1474	1785	7340
$AUC_{(0-\infty)}$	ng eq-hr/mL	3353	1519	1894	7796
$T_{1/2}$	hr	11.3	2.6	7.7	17.5
Excretion $urine_{(0-48h)}$	% of dose	0.83	0.42	0.35	2.00
Excretion $faeces_{(0-48h)}$	% of dose	0.042	0.04	0.003	0.163
Excretion $total_{(0-48h)}$	% of dose	0.87	0.46	0.35	2.16

These results indicate a C_{max} of 99.1 $\pm$ 38.4 ng_{eq}/mL and 266 $\pm$ 145 ng_{eq}/mL for the low and high concentration mixtures, respectively. $T_{1/2}$ was similar in both treatment groups (10.1 $\pm$ 1.3 hr and 11.3 $\pm$ 2.6 hr for the low and high concentration mixtures, respectively). The mean $AUC_{(0-\infty)}$ was 1241 $\pm$ 402 ng_{eq} x hr/mL and 3553 $\pm$ 1519 ng_{eq} x hr/mL for the low and high concentration mixtures, respectively, and these values were used in the MoS calculation.

Ref.: 15

Comment

The parent compound and its metabolites were not analyzed in urinary samples. Plasma half-lives of the test substance in the high dose group differed by 2.3-fold, in the low dose group by 1.5-fold suggesting that individual differences in the rate-limiting step(s) of elimination (metabolism and/or excretion) apparently do not play a major role. However, data on the N-acetylator status (NAT1, NAT2) of the study participants was not reported. Furthermore, in the high dose group, excretion in the 48 hour urine and faeces (combined) and C_{max} values differed by about 6-fold. In the low dose group, the differences were less marked (about 3-fold). In both dose groups, the AUC values differed by about 4-fold. The high dermal absorption in some cases (both high C_{max} and AUC values in 2 individuals in the high dose group and in 1 individual in the low dose group) were explained as follows: "The variability observed in the individual results is likely due to the variation in plasma levels that is frequently observed in studies performed via topical application." However, such differences may become crucial when the calculation of a toxicokinetic MoS gives a borderline result.

Conclusion on toxicokinetics

In an *in vitro* metabolism study with primary hepatocytes of human, rat and mouse toluene-2,5-diamine sulfate was extensively metabolized by all hepatocytes (order: rat ≈ mouse > human). With human hepatocytes the substance was completely metabolized after 4 h. For all three species N-acetylation was the major metabolic reaction and the results indicate that toluene-2,5-diamine sulfate is substrate for both types on N-acetyltransferases NAT1 and NAT2. Only a mono-N-acetylated metabolite was detected under the conditions of the study. No conclusions can be drawn from the study results with regards to the relative

activity or preference of human NAT1 and NAT2 towards the substrate toluene-2,5-diamine. Mouse hepatocytes additionally showed extensive hydroxylation of toluene-2,5-diamine sulfate.

The human hepatic metabolism *in vitro* of toluene-2,5-diamine sulfate was investigated in hepatocytes from human donors, pooled human liver microsomes and bacterially expressed human CYP isozymes. With microsomes there was no evidence for the production of oxidative metabolites. Incubation with recombinant CYP isozymes yielded no oxidative metabolites. Following incubation with human hepatocytes an indication was found by MS for the formation of a mono-acetylated derivative. No glucuronides, sulphate esters or mono-hydroxylated metabolites were detected.

Absorption, distribution, metabolism and excretion (ADME) after a single oral or dermal dose was studied in Kyoto rats, a strain with a slow acetylator phenotype. After oral dosing three metabolites were observed in the urine, the largest peak was identified as N,N-diacetyl-toluene-2,5-diamine which was also found in faeces. The metabolite pattern after dermal dosing was similar to that after oral dosing. The comparison of AUCs showed differences between oral (25 mg/kg bw: 174 mg_{eq} x h/l) and dermal application (33 mg/kg bw: 4.39 mg_{eq} x h/l). Following iv application, in urine 2 major metabolites were observed and the largest peak was identified as N,N-diacetyl-toluene-2,5-diamine which was also found in faeces. The metabolite pattern was similar to that after oral administration. The bioavailability (derived from comparison to iv administration) after oral dosing was > 90% while 2% bioavailability was found after dermal application.
In similar toxicokinetic studies with Sprague-Dawley rats the comparison of AUCs showed differences between oral (25 mg/kg bw: 112 mg_{eq} x h/l) and dermal application (33 mg/kg bw in formulation: 2.27 mg_{eq} x h/l). Following oral administration of 2.5 mg/kg bw (NOAEL dose) the AUC was 8.53 mg_{eq} x h/l. The bioavailability (derived from comparison to iv administration) after oral dosing was 69% while 2% bioavailability was found after dermal administration in a formulation.

Absorption, disposition and elimination in humans was studied following application of a hair dye formulation at a concentration of 0.825% toluene-2,5-diamine sulfate to the scalp. The exposed area was 250 cm^2. Following application of formulation I (non-oxidative conditions), the sum of excreted radioactivity in urine (0-48 h) was 2.31% (8.55 mg of 370 mg applied) whereas the respective figure for formulation II (oxidative conditions) was 0.78% (2.89 mg of 370 mg applied). With a H_2O_2 containing formulation, the mean absorption rate was 20 ± 2.02 $\mu g_{eq}/cm^2$ and the AUC was 9.2 ± 3.1 ng_{eq} x h/mL. This study was performed using a concentration of 0.825% on the scalp. However, the applicant intends the use of an on-head concentration of 3.6% toluene-2,5-diamine sulfate. The value of the sum of excreted radioactivity in urine determined was 2.89 mg toluene-2,5-diamine sulfate (= 1.6 mg toluene-2,5-diamine). Similarly, in the study of Schettgen et al. (2011) which investigated exposure to 2 commercial hair dye products in two females (both genotyped as slow acetylators with regard to NAT2), the cumulative urinary excreted amounts of toluene-2,5-diamine after collection for a time period of 48 h were 700 µg and 1500 µg, respectively. The kinetics of urinary toluene-2,5-diamine after dermal application showed a phase of absorption and distribution within the body of about 12 h. Excretion mainly followed a first-order kinetics with a urinary half-life of about 8 hours.

Studies added in submission III and IV

A further percutaneous absorption study was provided in submission III of 2010. The skin absorption of toluene-2,5-diamine sulfate at concentrations of 0.25, 0.8, 2.4, or 7.2% was investigated with human skin (abdomen, breast, back, thickness ca 400 µm). An area dose of 20 mg/cm^2 of the final formulation was applied once to the skin (0.64 cm^2) in a commercial oxidative hair dye formulation in either the presence or absence of hydrogen peroxide for 30 min. The study was well performed. Therefore, mean +1SD may be used as

the amount absorbed in calculating the MOS. The intended use of toluene-2,5-diamine is at a maximum of 4% 'on head' (7.2% as sulfate). Experiments were conducted with toluene-2,5-diamine sulfate in a range of dilutions from 0.25 to 7.2%. For 7.2% toluene-2,5-diamine sulfate, the amount absorbed under non-oxidative conditions (mean + 1SD) is 137.7 µg/cm². Under oxidative conditions, the amount absorbed is 69.4 µg/cm², which may be used for MOS calculation.

A further toxicokinetic study was conducted in female Sprague Dawley rats in accordance with OECD Guidelines 417 and 427 to determine plasma AUC of toluene-2,5-diamine sulfate equivalents after dermal application. The purpose of this study was to evaluate the concentration dependency of percutaneous absorption of toluene-2,5-diamine under *in vivo* conditions. The analysis of the results obtained in the four dose groups involving a 20 cm^2 dosing surface indicates that mean plasma $AUC_{(0-\infty)}$ was directly proportional to applied dose (expressed as µg/cm^2), and thus proportional to concentration used.

An additional rat oral toxicokinetic study was conducted in order to determine systemic exposure after a single dose of either 5 or 10 mg/kg bw toluene-2,5-diamine sulfate. The latter dose corresponds to the NOAEL determined in the new 91-day oral toxicity study. The $AUC_{(0-\infty)}$ value obtained in the 10 mg/kg bw dose group is considered to represent the systemic exposure at the dose corresponding to the NOAEL from the 91-day oral toxicity study.

Male and female animals (6 per sex per dose) were administered an oral gavage dose of [^{14}C]-toluene-2-5-diamine sulfate. Blood samples were collected at regular intervals over a 72-hour period, and radioactivity was measured in plasma. Toxicokinetic parameters including $AUC_{(0-\infty)}$ were calculated from the plasma concentration *versus* time profiles.

Toluene-2,5-diamine sulfate was rapidly absorbed following an oral dose, with a T_{max} of approximately 0.5 hr. C_{max} values corresponding to a dose of 10 mg/kg bw were 12.6 $\pm$ 1.11 μg_{eq} x h/ml and 13.3 $\pm$ 0.984 μg_{eq} x h/ml in males and females, respectively. Mean plasma $AUC_{(0-\infty)}$ values corresponding to a dose of 10 mg/kg bw were 41.0 $\pm$ 3.30 μg_{eq} x h/ml and 51.6 μg_{eq} x h/ml in males and females, respectively. The overall mean plasma $AUC_{(0-\infty)}$ for males and females combined was 46.3 μg_{eq} x h/ml, and this value was used by the applicant in the proposed toxicokinetics based MoS calculation.

In a further study, human systemic exposure (32 persons) was determined following application of an oxidative hair colouring formulation containing 1.5 and 4.0% toluene-2,5-diamine, respectively. The mean plasma $AUC_{(0-\infty)}$ values were 1241 $\pm$ 402 ng x hr/mL and 3553 $\pm$ 1519 ng x hr/mL for the low and high concentration mixtures, respectively. These values were used for interpolation of 2% toluene-2,5-diamine in order to calculate the MoS.
However, no data on the N-acetylator status (NAT1, NAT2) of the study participants were provided and, furthermore, marked individual differences in dermal absorption, C_{max} and AUC values were found.

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

See point 3.3.3. Sensitisation

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Toluene-2,5-diamine sulfate
(Oxidative conditions)

A. Conventional method

Absorption through the skin	**A (mean + 1SD)**	**=**	**40.02 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**23.21 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.39 mg/kg bw**
No observed adverse effect level (90-day, rat, oral)	**NOAEL**	**=**	**10 mg/kg bw/d**
NOAEL corrected for bio-availability (69%)		**=**	**6.9 mg/kg bw/d**
Margin of Safety	**NOAEL / SED**	**=**	**18**

B. Based on toxicokinetics

Following the SCCS Notes of Guidance, available oral absorption data and the toxicokinetic properties of the substance should be taken into account. According to WHO, the 100-fold uncertainty factor can be subdivided in interspecies differences (10-fold) in toxicodynamics (2.5) and toxicokinetics (4) and inter-individual differences (10-fold) in toxicodynamics (3.2) and toxicokinetics (3.2). Given the AUC figures obtained from rats and humans the 4-fold factor for interspecies differences in toxicokinetics can be set to 1 which results in a remaining uncertainty factor of 25 which should be achieved. The applicant proposed to use the new human exposure study *in vivo* to calculate the Margin of Safety using the area under curve. This approach is accepted as being scientifically valid.

In this new study human systemic exposure was determined following application of an oxidative hair colouring formulation containing 1.5 and 4.0% toluene-2,5-diamine, respectively. The mean plasma $AUC_{(0-\infty)}$ values were 1241 ng_{eq}-hr/mL and 3553 ng_{eq}-hr/mL for the low and high concentration mixtures, respectively. These values were compared to the rat mean $AUC_{(0-\infty)}$ of 46.3 μg_{eq}-hr/mL (see Ref. 14) at the NOAEL dose of 10 mg/kg bw/day established in the 91 day oral toxicity study. The toxicokinetics-based Margin of Safety was 37.3 and 13.0, respectively. By interpolation assuming direct proportionality the human plasma $AUC_{(0-\infty)}$ value corresponding to a MoS of 25 was calculated to be 1852 ng eq-hr/mL which corresponds to a concentration of 2.24% derived from the 1.5% AUC and assuming again proportionality. Therefore, the applied concentration of 2% is considered to be safe with regard to systemic toxicity (toxicokinetics based MoS >25). Furthermore, the intermittent exposure from oxidative hair dyes has to be taken into account.

3.3.14. Discussion

Physico-chemical properties
Toluene-2,5-diamine and its sulfate are used as an oxidative hair colouring agent (precursor). The intended maximum on-head concentration is 2.0% (calculated as free

base), or 3.6% (calculated as sulfate). Not all batches used were properly characterised. The stability of toluene-2,5-diamine and its sulfate in typical hair dye formulations was not reported. The impurity o-toluidine is classified by the EU as carcinogenic category 1B. No documentation was provided to support the reported data on the free base. Solubility of toluene-2,5-diamine has not been determined according to standard methods (e.g. EU - A.6).

Irritation / sensitisation
In an *in vivo* study in rabbits a 50.6% toluene-2,5-diamine applied under semi-occlusive conditions did not produce evidence of oedema and could not be evaluated for erythema due to black colouration of the skin. In the second experiment, which did not conform to guidelines or GLP, it is unclear whether the test concentration of toluene-2,5-diamine was 2.5% w/v or 25% w/v. However, in this experiment the test substance was irritant to rabbit skin under occlusive conditions.
Eye irritation studies have demonstrated that 50.6% toluene-2,5-diamine is irritant to the rabbit eye. Some irritant effects were also seen with 2.5% toluene-2,5-diamine.

The animal data indicate that toluene-2,5-diamine is an extreme skin sensitiser.
Human data: the results from several diagnostic patch studies in dermatitis patients show a high rate of contact allergy to toluene-2,5-diamine and to toluene-2,5-diamine sulfate. Due to different selection criteria and different patch test substances used, conclusions cannot be drawn concerning the trend over time of contact allergy to toluene-2,5-diamine and toluene-2,5-diamine sulfate. The results indicate that patch test reactivity is higher to toluene-2,5-diamine than toluene-2,5-diamine sulfate.

Dermal absorption
The data obtained in the different percutaneous absorption studies *in vitro* vary in the range of approximately 10 to 70 µg/cm^2. Such a variation is expected in view of the differences in design and data evaluation across studies. Factors which influenced the results were related to the test formulation (test substance concentration, presence of hydrogen peroxide and reaction partner, vehicle) to test model details (species/source and skin type) and to the number and type of compartments included in the calculation of systemic exposure.

A further percutaneous absorption study *in vitro* was provided in submission III of 2010. The skin absorption of toluene-2,5-diamine sulfate was investigated with human skin (abdomen, breast, back, thickness ca 400 µm). An area dose of 20 mg/cm² of the final formulation was applied once to the skin (0.64 cm²) in a commercial oxidative hair dye formulation in either the presence or absence of hydrogen peroxide for 30 min. The study was well performed. Therefore, mean +1SD may be used for MOS calculation. The intended use of toluene-2,5-diamine is at a maximum of 4% (calculated as free base) or 7.2% (calculated as sulfate) 'on head'. Experiments were conducted in a range of dilutions from 0.25 to 7.2% toluene-2,5-diamine sulfate. For 2.4% toluene-2,5-diamine sulfate, the amount absorbed under non-oxidative conditions (mean + 1SD) is 9.25 ± 2.54 = 11.79 µg/cm². Under oxidative conditions, the amount absorbed is 21.81 ± 4.87 = 26.68 µg/cm². The latter value may be used for the conventional MOS calculation after correction (26.68 x 3.6 / 2.4 = 40.02 µg/cm²) since the intended on head concentration is 3.6%.

General toxicity
The acute median lethal oral dose was calculated to be 102 mg/kg bw. In a 90-day study, the NOAEL is considered to be 10 mg/kg bw/d based on an increase in AST levels and myocyte degeneration observed at 20 mg/kg bw/d.

The NOAEL for reproductive toxicity was 45 mg/kg bw/d. The NOAEL of embryotoxicity and teratogenicity of toluene-2,5-diamine sulfate in rabbits is 50 mg/kg bw/d. The NOAEL of

toluene-2,5-diamine sulfate in rats for maternal toxicity is 50 mg/kg bw/d, the NOAEL of embryotoxicity and teratogenicity 80 mg/kg bw/d.

Toxicokinetics

In an *in vitro* metabolism study with primary hepatocytes of human, rat and mouse toluene-2,5-diamine sulfate was extensively metabolized by the hepatocytes of all species investigated (order: rat ≈ mouse > human). With human hepatocytes the substance was completely metabolized after 4 h. For all three species N-acetylation was the major metabolic reaction and the results indicate that toluene-2,5-diamine sulfate is substrate for both types on N-acetyltransferases NAT1 and NAT2. Only the mono-N-acetylated metabolite was detected under the conditions of the study. No firm conclusions can be drawn from the study results with regards to the relative activity or preference of human NAT1 and NAT2 towards the substrate toluene-2,5-diamine. Mouse hepatocytes additionally showed extensive hydroxylation of toluene-2,5-diamine sulfate.

The human hepatic metabolism *in vitro* of toluene-2,5-diamine sulfate was investigated in hepatocytes from human donors, pooled human liver microsomes and bacterially expressed human CYP isozymes. With microsomes there was no evidence for the production of oxidative metabolites. Incubation with recombinant CYP isozymes yielded no oxidative metabolites. Following incubation with human hepatocytes, an indication was found for the formation of a mono-acetylated derivative using mass spectrometry. No glucuronides, sulphate esters or mono-hydroxylated metabolites were detected.

Absorption, distribution, metabolism and excretion after a single oral or dermal dose was studied in Kyoto rats, a strain with a slow acetylator phenotype. After oral dosing three metabolites were observed in the urine, the largest peak was identified as N,N-diacetyl-toluene-2,5-diamine which was also found in faeces. The metabolite pattern after dermal dosing was similar to that after oral dosing. The comparison of AUCs showed differences between oral (25 mg/kg bw: 174 mg_{eq} x h/l) and dermal application (33 mg/kg bw: 4.39 mg_{eq} x h/l). Following iv administration, 2 major metabolites in urine were observed. The largest peak was identified as N,N-diacetyl-toluene-2,5-diamine which was also found in faeces. The metabolite pattern was similar to that after oral administration. The bioavailability (derived from comparison to iv administration) after oral dosing was > 90% while 2% bioavailability was found after dermal application.
In similar toxicokinetic studies with Sprague-Dawley rats the comparison of AUCs showed differences between oral (25 mg/kg bw: 112 mg_{eq} x h/l) and dermal application (33 mg/kg bw in formulation: 2.27 mg_{eq} x h/l). Following 2.5 mg/kg bw the AUC was 8.53 mg_{eq} x h/l. The bioavailability (derived from comparison to iv administration) after oral dosing of 10 mg/kg bw was 69% while 2% bioavailability was found after dermal administration in a formulation.

Absorption, disposition and elimination in humans was studied following application of a hair dye formulation at a concentration of 0.825% toluene-2,5-diamine sulfate to the scalp. The exposed area was 250 cm^2. Following application of a non-oxidative formulation, the sum of excreted radioactivity in urine (0-48 h) was 2.31% (8.55 mg of 370 mg applied) whereas the respective figure for an oxidative formulation was 0.78% (2.89 mg of 370 mg applied). With the H_2O_2 containing formulation, the mean absorption rate was 20 ± 2.02 $\mu g_{eq}/cm^2$ and the AUC was 9.2 ± 3.1 ng_{eq} x h/mL. This study was performed using a concentration of 0.825% on the scalp, while the applicant intends the use of an on-head concentration of 3.6% toluene-2,5-diamine sulfate.
In a recent study in in two females (both genotyped as slow acetylators with regard to NAT2), (Schettgen et al., 2011) a cumulative excretion of 700 to 1500 µg toluene-2,5-diamine after exposure to two different commercial hair dye products with unknown toluene-2,5-diamine content was found. The kinetics of urinary toluene-2,5-diamine after dermal application showed a phase of absorption and distribution within the body of about

12 h. Excretion mainly followed a first-order kinetics with a urinary half-life of about 8 hours.

A further toxicokinetic study was conducted in female Sprague Dawley rats in accordance with OECD Guidelines 417 and 427 to determine plasma AUC of toluene-2,5-diamine sulfate equivalents after dermal application. The purpose of this study was to evaluate the concentration dependency of percutaneous absorption of toluene-2,5-diamine under *in vivo* conditions. The analysis of the results obtained in the four dose groups involving a 20 cm^2 dosing surface indicates that mean plasma $AUC_{(0-\infty)}$ was directly proportional to applied dose (expressed as $\mu g/cm^2$), and thus proportional to concentration used.

An additional rat oral toxicokinetic study was conducted in order to determine systemic exposure after a single dose of either 5 or 10 mg/kg bw toluene-2,5-diamine sulfate. The latter dose corresponds to the NOAEL determined in the new 91-day oral toxicity study. The $AUC_{(0-\infty)}$ value obtained in the 10 mg/kg bw dose group is considered to represent the systemic exposure at the dose corresponding to the NOAEL from the 91-day oral toxicity study. Male and female animals (6 per sex per dose) were administered an oral gavage dose of [^{14}C]-toluene-2-5-diamine sulfate. Blood samples were collected at regular intervals over a 72-hour period, and radioactivity was measured in plasma. Toxicokinetic parameters including $AUC_{(0-\infty)}$ were calculated from the plasma concentration *versus* time profiles. Toluene-2,5-diamine sulfate was rapidly absorbed following an oral dose, with a T_{max} of approximately 0.5 hr. C_{max} values corresponding to a dose of 10 mg/kg bw were 12.6 $\pm$ 1.11 μg_{eq} x h/ml and 13.3 $\pm$ 0.984 μg_{eq} x h/ml in males and females, respectively. Mean plasma $AUC_{(0-\infty)}$ values corresponding to a dose of 10 mg/kg bw were 41.0 $\pm$ 3.30 μg_{eq} x h/ml and 51.6 μg_{eq} x h/ml in males and females, respectively. The overall mean plasma $AUC_{(0-\infty)}$ for males and females combined was 46.3 μg_{eq} x h/ml, and this value was used in the toxicokinetics-based MoS calculation.

In a further study human systemic exposure was determined following application of an oxidative hair colouring formulation containing 1.5 and 4.0% toluene-2,5-diamine, respectively. The mean plasma $AUC_{(0-\infty)}$ values were 1241 ± 402 ng_{eq}-hr/mL and 3553 $\pm$ 1519 ng_{eq}-hr/mL for the low and high concentration mixtures, respectively. These values were used in the toxicokinetics-based MoS calculation.

The knowledge on the metabolism of toluene-2,5-diamine in human skin and in systemic circulation is limited. From an *in vitro* study using human hepatocytes it was assumed by the study authors that NAT1 and NAT2 share the N-acetylation of toluene-2,5-diamine but the evidence is considered weak. Depending on the model system used, mono- and di-N-acetylated conjugates of toluene-2,5-diamine were detected, the di-conjugate mostly as the major metabolite. In human *in vivo* studies, the parent compound and its metabolites were not analyzed in urinary samples. From a scientific point of view, the role of N-acetylation of toluene-2,5-diamine by human NAT1 in human skin as a possible major metabolic inactivation step should be further investigated as well as polymorphisms of human NAT1.

Mutagenicity / genotoxicity

Overall, the genotoxicity of toluene-2,5-diamine sulfate is sufficiently investigated for the three types of mutation: gene mutation, structural chromosome mutation and aneuploidy.

Toluene-2,5-diamine sulfate is genotoxic *in vitro* inducing gene mutations in bacteria but not in mammalian cells, chromosomal aberrations, and unscheduled DNA-repair synthesis in primary hepatocytes *in vitro*.

The positive *in vitro* results could not be confirmed in *in vivo* experiments covering the same endpoints. Toluene-2,5-diamine sulfate was negative in two mouse bone marrow micronucleus tests, following oral and i.p. administration and in an *in vivo* UDS test following oral administration. The results of the *in vivo* Comet assay (oral gavage) in mice and rats in all organs evaluated except for the rat stomach may confirm the lack of genotoxic activity of toluene-2,5-diamine sulfate *in vivo*. However, issues with regard to interpretation and validity of the *in vivo* Comet assay in general and of the positive result in

the rat stomach in particular remain. In addition, toluene-2,5-diamine sulfate was negative in two dominant lethal assays indicating lack of genotoxic activity in germ cells *in vivo*. The negative results in two *in vivo* mouse spot tests following dermal and *ip* administration may confirm the lack of potential of toluene-2,5-diamine sulfate to induce gene mutations.
As the clastogenic effects found *in vitro* were not confirmed in *in vivo* tests, toluene-2,5-diamine sulfate can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
Toluene-2,5-diamine sulfate has been studied for carcinogenicity after oral administration by US National Cancer Institute. The conclusion drawn in the NCI report for this study was: *"under the conditions of this bioassay, sufficient evidence was not provided to conclusively demonstrate the carcinogenicity of 2,5-toluenediamine sulfate in either Fischer 344 rats or B6C3F1 mice".* Hair dye formulations of toluene-2,5-diamine together with hydrogen peroxide have been tested in three experimental studies after topical application to mice or rats. The sensitivity of these studies may have been low as no response to hair dye formulations containing known carcinogens was observed. Thus, no conclusions with regard to carcinogenicity can be drawn from the skin painting studies.

Margin of Safety
Toxicokinetic data for Sprague-Dawley rats indicate that oral availability of toluene-2,5-diamine at 10 mg/kg bw is 69%. The NOAEL from a 90-day study (10 mg/kg bw/d) was corrected accordingly to 6.9 resulting in a MOS of 18.

The applicant proposed to use the new human exposure study *in vivo* to calculate the Margin of Safety using the area under curve. This approach is accepted as being scientifically valid. The SCCS favours a toxicokinetics-based Margin of Safety. In a new study human systemic exposure was determined following application of an oxidative hair colouring formulation containing 1.5 and 4.0% toluene-2,5-diamine, respectively. The mean plasma $AUC_{(0-\infty)}$ values were 1241 ng eq-hr/mL and 3553 ng eq-hr/mL for the low and high concentration mixtures, respectively. These values were compared to the rat mean $AUC_{(0-\infty)}$ of 46.3 µg eq-hr/mL (see Ref. 14) at the NOAEL dose of 10 mg/kg bw/day established in the 91 day oral toxicity study. The toxicokinetics-based Margin of Safety was 37.3 and 13.0, respectively. By interpolation assuming direct proportionality the human plasma $AUC_{(0-\infty)}$ value corresponding to a MoS of 25 was calculated to be 1852 ng eq-hr/mL which corresponds to a concentration of 2.24% derived from the 1.5% AUC and assuming again proportionality. Therefore, the applied concentration of 2% is considered to be safe with regard to systemic toxicity (toxicokinetics based MoS >25).

4. CONCLUSION

Using the conventional risk assessment approach, a Margin of Safety of 18 was calculated.

However, using a toxicokinetics-based approach based on a new human exposure study *in vivo* the Margin of Safety was calculated using the area under the curve. The applied concentration on-head of maximum 2% (calculated as free base) or 3.6% (calculated as sulfate salt) is considered to be safe with regard to systemic toxicity (toxicokinetics based MoS >25).

Toluene-2,5-diamine is an extreme skin sensitiser. The frequency of allergic reactions in hairdressers and consumers remains a considerable concern for consumer safety.

5. MINORITY OPINION

Not applicable

6. REFERENCES

1. First report on the harmonisation of risk assessment procedures (Chapter 9 - Risk Characterisation); EUROPEAN COMMISSION; 95-108; 2000
2. Contrera, J. F.; Jacobs, A. C.; Prasanna, H. R.; Mehta, M.; Schmidt, W. J.; De George, J.; A systemic exposure-based alternative to the maximum tolerated dose for carcinogenicity studies of human therapeutics; J. AM. COLL. TOXICOL.; 14, 1-10; 1995
3. Damkröger, G.; Identity, stability and purity test of 1,4-Diamino-2-methyl-benzene-sulphate (1:1); WELLA AG; 2000
4. Lange, J.; Sieve analysis; NOACK; 2004
5. Franke, J.; Particle size distribution; SIEMENS AXIVA GMBH & CO. KG; 2004
6. Lange, J.; Water solubility (Flask Method); NOACK; 2004
7. Lange, J.; Prediction of the pKa-value; NOACK; 2004
8. Smeykal, H.; Melting point / Boiling point; SIEMENS AXIVA GMBH & CO. KG; 2004
9. Lange, J.; Determination of the density; NOACK; 2004
10. Franke, J.; Vapour pressure; SIEMENS AXIVA GMBH & CO. KG; 2004
11. Lange, J.; Surface tension; NOACK; 2004
12. Lange, J.; Flammability of solids; NOACK; 2004
13. Smeykal, H.; Auto-Flammability (Solids - Determination of relative self-ignition temperature); SIEMENS AXIVA GMBH & CO. KG; 2004
14. Opinion of A005; SCCNFP; 1985
15. Final report on the safety assessment of Toluene-2,5-diamine, Toluene-2,5-diamine sulfate, and Toluene-3,4-diamine; J. AM. COLL. TOXICOL.; 11, 423-445; 1992
16. Kynoch, S. R.; Lloyd, G. K.; Acute oral toxicity to rats of Toluene-2,5-diamine; HUNTINGDON; 1975
17. de Jouffrey, S.; Acute dermal irritation in rabbits; CENTRE INTERNATIONAL DE TOXICOLOGIE; 1994
18. Kynoch, S. R.; Liggett, M. P.; Irritant effects of Toluene-2,5-diamine on rabbit skin; HUNTINGDON; 1975
19. Clouzeau, J.; Acute eye irritation in rabbits; CENTRE INTERNATIONAL DE TOXICOLOGIE; 1994
20. Kynoch, S. R.; Liggett, M. P.; Irritant effects of Toluene-2,5-diamine on rabbit eye mucosa; HUNTINGDON; 1975
21. Schröder, K.; In-vitro-test: HET-CAM [HCI]- End-Point assessment - „p-Toluylendiamin sulfat - Colipa A5 - pTD"; HENKEL; 2002
22. Aeby, P.; Wyss, C.; Beck, H.; Griem, P.; Scheffler, H.; Goebel, C.; Characterization of the sensitizing potential of chemicals by in vitro analysis of dendritic cell activation and skin penetration; J. INVEST. DERMATOL.; 122, 1154-1164; 2004
23. Contact sensitisation: Classification according to potency; ECETOC; 2003
24. Albrecht, A.; Test for Sensitization (Local Lymph Node Assay - LLNA) with Haarbraun; BIOSERVICE; 2001, No. 000322D
25. Albrecht, A.; Test for sensitization (Local Lymph Node Assay - LLNA) with Haarbraun; BIOSERVICE; 2001, No. 000322H
26. Xie, Z.; Hayakawa, R.; Sugiura, M.; Kojima, H.; Konishi, H.; Ichihara, G.; Takeuchi, Y.; Experimental study on skin sensitization potencies and cross-reactivities of hair-dye-related chemicals in guinea pigs; CONTACT DERMATITIS; 42, 270-275; 2000
27. Ishihara, M.; Nogami, T.; Itoh, M.; Nishimura, M.; Sensitization potency of dye intermediates and modifiers in guinea pigs; SKIN RES.; 27, 585-590; 1985
28. Leclerc, C.; In vitro transcutaneous absorption of Toluene 2,5-diamine sulfate; L'OREAL; 1997

29. Bornatowicz, N.; Dermal absorption / percutaneous penetration of "pTD-sulfate" in vitro; SEIBERSDORF; 2002
30. Wyss, C.; Cutaneous absorption of Toluene-2,5-diamine sulfate in formulation through pig skin in vitro; COSMITAL; 2004
31. Wyss, C.; Cutaneous absorption of 2-Methyl-1,4-benzenediamine sulphate (Haarbraun) in Aceton/Aqua/Olive Oil (2:2:1) through pig skin in vitro; COSMITAL; 2001
32. Hill, R. E.; Paratoluenediamine 14 day oral (gavage) rangefinding study in the rat; TOXICOL LAB. LTD.; 1994
33. Hill, R. E.; Paratoluenediamine 13 week oral (gavage) toxicity study in the rat; QUINTILES ENGLAND LTD; 1997
34. Sokolowski, A.; Salmonella Typhimurium reverse mutation assay with INCI: Toluene-2,5-diamine sulfate; RCC-CCR; 2003
35. Wollny, H.-E.; Cell mutation assay at the thymidine kinase (TK +/-) locus in mouse lymphoma L5178Y cells with A5; RCC-CCR; 1995
36. Schulz, M.; In vitro chromosome aberration test in Chinese hamster V79 cells with SAT 010935; RCC-CCR; 2002
37. Kornbrust, D. J.; Barfknecht, T. R.; Comparison of 7 azo dyes and their azo reduction products in the rat and hamster hepatocyte primary culture/DNA-repair assays; MUTATION RESEARCH; 136, 255-266; 1984
38. Bornatowicz, N.; "SAT 010935": Micronucleus test with mice; SEIBERSDORF; 2002
39. Völkner, W.; Micronucleus assay in bone marrow cells of the mouse with A 5; RCC-CCR; 1995
40. Getuli, C.; SAT 010935 Unscheduled DNA synthesis (UDS) in primary rat hepatocytes after in vivo treatment; RTC; 2002
41. Cinelli, S.; SAT 010935 Unscheduled DNA synthesis (UDS) in primary rat hepatocytes after in vivo treatment; RTC; 2004
42. Hartmann, A.; Agurell, E.; Beevers, C.; Brendler-Schwaab, S.; Burlinson, B.; Clay, P.; Collins, A.; Smith, A.; Speit, G.; Thybaud, V.; Tice, R. R.; Recommendations for conducting the in vivo alkaline comet assay; MUTAGENESIS; 18, 45-51; 2003
43. Brendler-Schwaab, S.; Hartmann, A.; Pfuhler, S.; Speit, G.; The in vivo comet assay: use and status in genotoxicity testing; MUTAGENESIS; 20, 245-254; 2005
44. Sekihashi, K.; Yamamoto, A.; Matsumura, Y.; Ueno, S.; Watanabe-Akanuma, M.; Kassie, F.; Knasmüller, S.; Tsuda, S.; Sasaki, Y. F.; Comparative investigation of multiple organs of mice and rats in the comet assay; MUTATION RESEARCH; 517, 53-75; 2002
45. Sasaki, Y. F.; Sekihashi, K.; Izumiyama, F.; Nishidate, E.; Saga, A.; Ishida, K.; Tsuda, S.; The comet assay with multiple mouse organs: comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP Carcinogenicity Database; CRIT. REV. TOXICOL.; 30, 629-799; 2000
46. Soares, E. R.; Lock; L. F.; Lack of an indication of mutagenic effects of dinitrotoluenes and diaminotoluenes in mice; ENVIRON. MUTAGENESIS; 2, 111-124; 1980
47. Matheson, D. W.; Mutagenicity evaluation of Orex 111 in the somatic mutation assay; LITTON BIONETICS; 1978
48. Burnett, C.; Loehr, R.; Corbett, J.; Dominant Lethal mutagenicity study on hair dyes; J. TOXICOL. ENVIRON. HEALTH; 2, 657-662; 1977
49. Matheson, D. W.; Mutagenicity evaluation of 2,5-Diaminotoluene, dihydrochloride in the mouse dominant lethal assay; LITTON BIONETICS; 1978
50. Compendium of abstracts from long-term cancer studies reported by the National Toxicology Program from 1976 to 1992; NATIONAL INSTITUTE OF HEALTH; 101, 72-74; 1993
51. Bioassay of 2,5-Toluenediamine sulfate for possible carcinogenicity; NATIONAL INSTITUTE OF HEALTH; 1978
52. Bornatowicz, N.; Two-generation fertility study with p-Toluylenediamine sulphate in rats; SEIBERSDORF; 1986
53. Osterburg, I.; p-Toluylenediamine sulphate oral (gavage) teratology study in the New Zealand White rabbit; HAZLETON; 1982

54. Osterburg, I.; p-Toluylenediamine sulphate oral (gavage) teratology study in the rat; HAZLETON; 1982
55. Marks, T. A.; Gupta, B. N.; Ledoux, T. A.; Staples, R. E.; Teratogenic evaluation of 2-Nitro-p-phenylenediamine, 4-Nitro-o-phenylenediamine, and 2,5-Toluenediamine sulfate in the mouse; TERATOLOGY; 24, 253-265; 1981
56. Krebsfänger, N.; 2,4-Diaminotoluene sulfate (23005): Metabolic stability, metabolite profile, and species comparison in primary hepatocytes of human, rat, and mouse; GENPHARMTOX; 2003
57. Powrie, R.; Human hepatic metabolism of Toluene-2,5-diamine: in vitro analysis; CXR BIOSCIENCES LTD.; 2005
58. Wenker, M. A. M.; Absorption, distribution, metabolism and excretion of 14C-Toluene-2,5-diamine sulphate in the female Wistar Kyoto rat after a single oral or dermal dose; NOTOX; 2005
59. Wenker, M. A. M.; Absorption, distribution, metabolism and excretion of 14C-Toluene-2,5-diamine sulphate in the female Wistar Kyoto rat after a single intravenous dose; NOTOX; 2005
60. Wenker, M. A. M.; Pharmacokinetics of 14C-Toluene-2,5-diamine sulphate in the female Sprague-Dawley rat after a single oral or dermal dose; NOTOX; 2005
61. Wenker, M. A. M.; Pharmacokinetics of 14C-Toluene-2,5-diamine sulphate in the female Sprague-Dawley rat after a single intravenous dose; NOTOX; 2005
62. Aylward, M.; p-(Me-14C) Toluenediamine absorption through hair-bearing scalp, disposition & elimination in adult healthy subjects following application of two formulations containing p-Toluenediamine; SIMBEC RESEARCH LABORATORIES; 1981
63. Lynde, C. W.; Mitchell, J. C.; Patch test results in 66 hairdressers 1973-81; CONTACT DERMATITIS; 8, 302-307; 1982
64. Uter, W.; Geier, J.; Schnuch, A.; Current pattern and trends in sensitization to hairdressers allergens in Germany; OCCUP. ENVIRON. DERMATOL.; 48, 55-59; 2000
65. Katsarou, A.; Koufou, B.; Takou, K.; Kalogeromitros, D.; Papanayiotou, G.; Vareltzidis, A.; Patch test results in hairdressers with contact dermatitis in Greece (1985-1994); CONTACT DERMATITIS; 33, 347-348; 1995
66. Van der Walle, H. B.; Brunsveld, V. M.; Dermatitis in hairdressers (I). The experience of the past 4 years; CONTACT DERMATITIS; 30, 217-221; 1994
67. Frosch, P. J.; Burrows, D.; Camarasa, J. G.; Dooms-Goossens, A.; Ducombs, G.; Lahti, A.; Menne, T.; Rycroft, R. J. G.; Shaw, S.; White, I.; Wilkinson, J. D.; Allergic reactions to hairdresser`s series - results of nine European centers; EUROPEAN ENVIRONMENTAL AND CONTACT DERMATITIS RESE
68. Guerra, L.; Tosti, A.; Bardazzi, F.; Pigatto, P.; List, P.; Santucci, B.; Valsecchi, R.; Schena, D.; Angelini, G.; Sertoli, A.; Ayala, F.; Kokeli, F.; Contact dermatitis in hairdressers: the Italian experience; CONTACT DERMATITIS; 26, 101-107; 1992
69. Frosch, P. J.; Aktuelle Kontaktallergene; DER HAUTARZT; 41, 129-133; 1990
70. Toxicity profile Toluene-2,5-diamine (and its salts); TNO BIBRA INTERNATIONAL LTD; 1995
71. Holm, J. O.; Veierod, M. B.; An epidemiological study of hand eczema. V. Prevalence among hairdresser trainees, compared with a general population of hairdressers; ACTA DERM. VENEREOL.; 187, 23-25; 1994
72. Uter, W.; Lessmann, H.; Geier, J.; Schnuch, A.; Contact allergy to ingredients of hair cosmetics in female hairdressers and clients - an 8-year analysis of IVDK data; CONTACT DERMATITIS; 49, 236-240; 2003
73. Iorizzo, M.; Parente, G.; Vincenzi, C.; Pazzaglia, M.; Tosti, A.; Allergic contact dermatitis in hairdressers: frequency and source of sensitisation; EUR. J. DERMATOL.; 12, 179-182; 2002
74. Schwarz, A.; Gottmann-Lückerath, J.; Allergenhäufigkeit bei Kontaktallergien in der Universitäts-Hautklinik Köln (1970-1971 und 1976-1979); Z. HAUTKR.; 57, 951-960; 1981
75. Kuhlwein, A.; Hausen, B. M.; Standardepikutantestung: Statistische Auswertung aus fünf Jahren; Z. HAUTKR.; 57, 1179-1186; 1982

76. Broeckx, W.; Blondeel, A.; Dooms-Goossens, A.; Achten, G.; Cosmetic intolerance; CONTACT DERMATITIS; 16, 189-194; 1987
77. Jarisch, R.; Sandor, I.; Epikutanstandardtestung: Ergebnisse aus fünf Jahren und ihre Auswirkungen auf zukünftige Untersuchungen; Z. HAUTKR.; 53, 462-470; 1978
78. Guerra, L.; Bardazzi, F.; Tosti, A.; Contact dermatitis in hairdressers´ clients; CONTACT DERMATITIS; 26, 108-111; 1992
79. Koopmans, A. K.; Bruynzeel, D. P.; Is PPD a useful screening agent?; CONTACT DERMATITIS; 48, 89-92; 2003
80. Goossens, A.; Beck, M. H.; Haneke, E.; McFadden, J. P.; Nolting, S.; Durupt, G.; Ries, G.; Adverse cutaneous reactions to cosmetic allergens; CONTACT DERMATITIS; 40, 112-113; 1999
81. Uter, W.; Lessmann, H.; Geier, J.; Becker, D.; Fuchs, T.; Richter, G.; The spectrum of allergic (cross-)sensitivity in clinical patch testing with 'para amino' compounds; ALLERGY; 57, 319-322; 2002
82. Kohl, L.; Blondeel, A.; Song, M.; Allergic contact dermatitis from cosmetics. Retrospective analysis of 819 patch-tested patients; DERMATOLOGY; 204, 334-337; 2002
83. Uter, W.; Lessmann, H.; Geier, J.; Becker, D.; Fuchs, Th.; Richter, G.; Die Epikutantestung mit "Parastoffen"; OCCUP. ENVIRON. DERMATOL.; 50, 97-104; 2002
84. Hamilton, S.; Sheridan, J.; Aplastic anaemia and hair dye; BR. MED. J.; 1, 834; 1976
85. Toghill, J.; Wilcox, G.; Aplastic anaemia and hair dye; BR. MED. J.; 1, 502-503; 1976
86. Hopkins, J. E.; Manoharan, A.; Severe aplastic anaemia following the use of hair dye: report of two cases and review of literature; POSTGRAD. MED. J.; 61, 1003-1005; 1985
87. König, P.; Data base search for references for A005 - Toluene-2,5-Diamine sulfate: 615-50-9 (sulfate[1:1]), 6369-59-1 (sulfate) parent substance: 2,5-Diaminotoluene: 95-70-5; WELLA AG; 2005
88. Burnett, C. M.; Goldenthal, E. I.; Multigeneration reproduction and carcinogenicity studies in Sprague-Dawley rats exposed topically to oxidative hair-colouring formulations containing p-phenylenediamine and other aromatic amines; *FD. CHEM. TOXICOL.*; *26*, *467-474*; 1988
89. Kinkel, H. J.; Holzmann, S.; Study of Long-term Percutaneous Toxicity and Carcinogenicity of Hair Dyes (Oxidizing Dyes) in Rats; *FD. COSMET. TOXICOL.*; *11*, *641-648*; 1973
90. Burnett, C.; Lanman, B.; Giovacchini, R.; Wolcott, G.; Scala, R.; Keplinger, M.; Long-term toxicity studies on oxidation hair dyes; *FD. COSMET. TOXICOL.*; *13*, *353 -357*; 1975
91. Giles, A. L.; Chung, Ch. W.; Dermal carcinogenicity study by mouse-skin painting with 2,4-toluenediamine alone or in representative hair dye formulations; *J. TOXICOL. ENVIRON. HEALTH*; *1*, *433-440*; 1976
92. Burnett, C.; Jacobs, M. M.; Seppala, A.; Shubik, P.; Evaluation of the toxicity and carcinogenicity of hair dyes; *J. TOXICOL. ENVIRON. HEALTH*; *6*, *247-257*; 1980
93. Potokar, M.; Pittermann, W.; Subakute orale Toxizität an Ratten bei einer Versuchsdauer von 12 Wochen; *HENKEL*; 1979
94. Mosesso, P.; Assessment of clastogenic action on bone marrow erythroblasts in the Chromosome Aberration test; *LSR*; 1979
95. Fautz, R.; In vivo/in vitro UDS Synthesis in rat hepatocytes with A 5; *CCR*; 1994
96. Chung, K.-T.; Murdock, C. A.; Stevens, S. E. Jr.; Li Y.-S.; Wei, C.-I.; Huang, T.-S.; Chou, M. W.; Mutagenicity and toxicity studies of p-phenylenediamine and its derivatives; *TOXICOL. LETT.*; *81*, *23-32*; 1995
97. Sasaki, Y. F.; Fujikawa, K. et. al.; The alkaline single cell gel electrophoresis assay with mouse multiple organs: results with 30 aromatic amines evaluated by the IARC and U.S. NTP.; *MUTATION RESEARCH*; *440*, *1-18*; 1999
98. Lloyd, G. K.; Liggett, M. P.; Kynoch, S. R.; Davies, R. E.; Assessment of the acute toxicity and potential irritancy of hair dye constituents; *FD. COSMET. TOXICOL.*; *15*, *607-610*; 1977

99. Burnett, C.; Goldenthal, E. I.; Harris, S. B.; Wazeter, F. X.; Strausburg, J.; Kapp, R.; Voelker, R.; Teratology and percutaneous toxicity studies on hair dyes; *J. TOXICOL. ENVIRON. HEALTH*; *1*, *1027-1040*; 1976
100. Kiese, M.; Rachor; Rauscher, E.; The absorption of some Phenylenediamines through the skin of dogs; *TOXICOL. APPL. PHARMACOL.; 12*, *495-507*; 1968
101. Hruby, R.; The absorption of p-Toluenediamine by the skin of rats and dogs; *FD. COSMET. TOXICOL.*; *15*, *595-599*; 1977
102. Kiese, M.; Rauscher, E.; The Absorption of p-Toluenediamine through human skin in hair dyeing; *TOXICOL. APPL. PHARMACOL.*; *13*, *325-331*; 1968
103. Ames, B. N.; Kammen, H. O.; Yamasaki, E.; Hair dyes are mutagenic: Identification of a variety of mutagenic ingredients; *PROC. NATL. ACAD. SCI.*; *72*, *2423-2427*; 1975
104. Hossack, D. J. N.; Richardson, J. C.; Examination of the potential mutagenicity of hair dye constituents using the micronucleus test ; *EXPERIENTIA*; *33*, *377-378*; 1977
105. Inouye, M.; Murakami, U.; Teratogenicity of 2,5-Diaminotoluene, a hair-dye constituent, in mice; *FD. COSMET. TOXICOL.*; *15*, *447-451*; 1977
106. Burnett, C.; Fuchs, C.; Corbett, J.; Menkart, J.; The effect of Dimethylsulfoxide on the mutagenicity of the hair dye p-Phenylenediamine; *MUTATION RESEARCH*; *103*, *1-4*; 1982
107. Spengler, J.; Osterburg, I.; Korte, R.; Teratogenic evaluation of p-Toluenediamine sulphates, Resorcinol and p-Aminophenol in rats and rabbits; *TERATOLOGY*; *33*, *1-4*; 1986
108. Hofer, H.; Bornatowicz, N.; Reindl, E.; Analysis of human chromosomes after repeated hair dyeing; *FD. CHEM. TOXICOL.*; *21*, *785-789*; 1983
109. L'Oreal, Aulnay-sous-bois, France; Etude comparative de la reactivité in vitro de cinq colorants capillaries vis-à-vis de mitochondrie isolees de muscles de rat; Report No: TM 96/001; 1996
110. Büsching, J.; Subakute Toxizitätsprüfung mit p-Toluylendiaminsulfat, p-Phenylendiamin und Resorcin nach oraler Applikation an Ratten; *WELLA AG*; 1981
111. Cheung, Y. L.; Snelling, J.; Mohammed. N. N.; Gray. T. J.; Ioannides. C.; Interaction with the aromatic hydrocarbon receptor, CYP1A induction, and mutagenicity of a series of diaminotoluenes: implications for their carcinogenicity; *TOXICOL. APPL. PHARMACOL.*; *139*, *203-211*; 1996
112. National Toxicology Program; Salmonella assay; Study ID 578137; 1985
113. National Toxicology Program; Mouse lymphoma assay; Aliquot No. 821795
114. National Toxicology Program; Chromosome aberration assay and sister chromatid exchange assay in vitro; Study No. 951026
115. TOLUENE-2,5-DIAMINE, 50% Aqueous solution: Evaluation of solubility at 1 g/100ml and 10 g/100ml, 2007

Submission III, October 2010

1. Weber, K. Peer Review to: Para-toluenediamine 13 Week Oral (Gavage) Toxicity Study in the Rat (LRL/42/94): Peer Review on Histopathology Examinations from Selected Organs and on Values Recorded for Hematology and Clinical Biochemistry. 12 Oct 2008
2. Prentice D.E. A Review of the Clinical Pathology of Paratoluenediamine (PTD) in a 13 Week Oral (Gavage) Study in the Rat (LRL/42/94). 7 November 2008
3. Prentice D.E.: Paratoluenediamine (PTD) 13 Week Oral (Gavage) Study in the Rat (LRL/42/94) – Histopathology Peer Review. 6 November 2008
4. Weber, K. Peer Review to: Para-toluenediamine 14 Day Oral (Gavage) Range-finding Study in the Rat (LRL/41/93): Peer Review on Histopathology Examinations from Selected Organs. 12 Oct 2008
5. Prentice D.E. Paratoluenediamine (PTD) 14 Day Oral (Gavage) Study in the Rat (LRL/41/93) – Histopathology Peer Review. 6 November 2008
6. Aylward, M. p-(Me-14C) Toluenediamine absorption through hair-bearing scalp, disposition & elimination in adult healthy subjects following application of two

formulations containing p-Toluenediamine; Simbec Research Laboratories (1981) [report previously provided in Submission II, 14 July 2005]
7. Hill, R.E. Paratoluenediamine 13 week oral (gavage) toxicity study in the rat: LRL/42/94 QUINTILES ENGLAND LTD; 1997 [report previously provided in Submission II, 14, July 2005]
8. Schettgen T, Heinrich K, Kraus T, and Gube M. Determination of 2,5-toluylenediamine (2,5-TDA) and aromatic amines in urine after personal application of hair dyes: kinetics and doses. Arch. Toxicol. In press. Published on line: 15 June 2010
9. IPCS. International Program on Chemical Safety. Chemical-specific adjustment factors for interspecies differences and human variability: guidance document for the use of data in dose/concentration-response assessment. Harmonization Project Document No. 2. World Health Organization, Geneva, Switzerland (2005)
10. WHO. Assessing human health risks of chemicals; derivation of guidance values for health-based exposure limits. Environmental Health Criteria, 170, WHO, Geneva (1994)
11. In vitro percutaneous absorption of Toluene-2,5-diamine sulfate through human skin membranes using flow-through diffusion cells. Study Number V8184, TNO Quality of Life, The Netherlands, June 2009.
12. Pharmacokinetics of 14C-toluene-2,5-diamine sulfate in the Sprague-Dawley rat after a single dermal dose at different concentrations. Project Number 488796, NOTOX, The Netherlands, June 2009
13. A 91-day toxicity study of toluene-2,5-diamine sulfate (WR 23005, A005) administered by oral gavage to rats with a 28-day recovery period. Study Number VKA00169, Charles River, Spencerville, Ohio, USA, August 2010
14. Toxicokinetics of [14C] toluene-2,5-diamine sulfate following a single oral administration to Sprague-Dawley rats. Study Number 420782, Charles River, Senneville, Quebec, Canada, September 2010
15. Consumer exposure to an oxidative hair dye. A [14C]-labelled Toluene-2,5-diamine mass balance study. Study Number V9317, TNO, Zeist, The Netherlands, January 2012

Additional references

AR1. Munday R, Manns E, Fowke EA, Hoggard GK (1990) Structure-activity relationships in the myotoxicity of ring-methylated p-phenylenediamins in rats and correlation with autoxidation rates in vitro. Chemical-Biological Interactions 76: 31-45
AR2. Munday R, Manns E (1999) Muscle necrosis in rats induced by 2-methoxy-p-phenylenediamine. Food and Chemical Toxicology 37: 561-564
AR3. Schettgen T, Heinrich K, Kraus T, Gube M (2011) Determination of 2,5-toluylenediamine (2,5-TDA) and aromatic amines in urine after personal application of hair dyes: kinetics and doses. Archives of Toxicology 85: 127-133

EXHIBIT E

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No. 126
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BIOASSAY OF 2,5-TOLUENEDIAMINE SULFATE FOR POSSIBLE CARCINOGENICITY

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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health

BIOASSAY OF

2,5-TOLUENEDIAMINE SULFATE

FOR POSSIBLE CARCINOGENICITY

Carcinogenesis Testing Program
Division of Cancer Cause and Prevention
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20014

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health

DHEW Publication No. (NIH) 78-1381

REPORT ON THE BIOASSAY OF 2,5-TOLUENEDIAMINE SULFATE FOR POSSIBLE CARCINOGENICITY

CARCINOGENESIS TESTING PROGRAM
DIVISION OF CANCER CAUSE AND PREVENTION
NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH

FOREWORD: This report presents the results of the bioassay of 2,5-toluenediamine sulfate conducted for the Carcinogenesis Testing Program, Division of Cancer Cause and Prevention, National Cancer Institute (NCI), National Institutes of Health, Bethesda, Maryland. This is one of a series of experiments designed to determine whether selected chemicals have the capacity to produce cancer in animals. Negative results, in which the test animals do not have a significantly greater incidence of cancer than control animals, do not necessarily mean the test chemical is not a carcinogen because the experiments are conducted under a limited set of circumstances. Positive results demonstrate that the test chemical is carcinogenic for animals under the conditions of the test and indicate a potential risk to man. The actual determination of the risk to man from animal carcinogens requires a wider analysis.

CONTRIBUTORS: This bioassay of 2,5-toluenediamine sulfate was conducted by Mason Research Institute, Worcester, Massachusetts, initially under direct contract to the NCI and currently under a subcontract to Tracor Jitco, Inc., prime contractor for the NCI Carcinogenesis Testing Program.

The experimental design was determined by the NCI Project Officers, Dr. J. H. Weisburger (1,2) and Dr. E. K. Weisburger (1). The principal investigators for the contract were Dr. E. Smith (3) and Dr. A. Handler (3). Animal treatment and observation were supervised by Mr. G. Wade (3) and Ms. E. Zepp (3).

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Compilation of individual animal survival, pathology, and summary tables was performed by EG&G Mason Research Institute (5); the statistical analysis was performed by Mr. W. W. Belew (6,7), using methods selected for the Carcinogenesis Testing Program by Dr. J. J. Gart (8).

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SUMMARY

A bioassay for possible carcinogenicity of 2,5-toluenediamine sulfate was conducted using Fischer 344 rats and B6C3F1 mice. 2,5-Toluenediamine sulfate was administered in the feed, at either of two concentrations, to groups of 50 males and 50 females of each species. The high and low time-weighted average concentrations of the compound were, respectively, 0.2 and 0.06 percent for rats and 0.1 and 0.06 percent for mice. Because compound administration to the high and low dose groups of each species was not begun simultaneously, each dosed group was assigned a control group. All control groups consisted of 50 animals, except for the high dose male and female rat control groups which were composed of 25 animals. The dosing period was for 78 weeks, followed by an additional 28 to 31 weeks of observation in rats and an additional 16 to 19 weeks in mice.

There was no significant association between compound administration and accelerated mortality in either sex of either species. Adequate numbers of animals in all groups survived sufficiently long to be at risk from late-developing tumors.

A statistically significant incidence of interstitial-cell neoplasms of the testis in dosed male rats was not considered attributable to administration of the compound since the spontaneous incidence of these neoplasms in male Fischer 344 rats is both high and variable. No neoplasms were observed in female rats at statistically significant incidences.

A statistically significant increase in lung tumors in high dose female mice was not considered convincing evidence of a compound-related carcinogenic effect because high dose mice were received in separate shipments from their controls and housed in separate rooms from their controls.

Under the conditions of this bioassay, sufficient evidence was not obtained to demonstrate the carcinogenicity of 2,5-toluenediamine sulfate in either Fischer 344 rats or B6C3F1 mice.

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I. INTRODUCTION

2,5-Toluenediamine sulfate (Figure 1) (NCI No. C01832), a salt of 2,5-toluenediamine and sulfuric acid, was selected for bioassay by the National Cancer Institute in an attempt to determine those dye intermediates which may be responsible for the increased incidence of bladder cancer observed among workers in the dye manufacturing industry (Wynder et al., 1963; Anthony and Thomas, 1970). Aromatic amines are one of several classes of chemicals thought to contribute to the increased cancer risk in this industry (Wynder et al., 1963).

The Chemical Abstracts Service (CAS) Ninth Collective Index (1977) name for this compound is 2-methyl-1,4-benzenediamine sulfate.* It is also called p-tolylenediamine sulfate; p-diaminotoluene sulfate; fouramine standard; and Colour Index (C.I.) oxidation base 4 (C.I. No. 76043).

2,5-Toluenediamine is used in the synthesis of safranine, a family of dyes, some of which are useful as biological stains (Hawley, 1971), and as an oxidation base to dye furs a deep brown (Society of Dyers and Colourists, 1971). In addition, 2,5-toluenediamine is a common component of the "permanent" or oxidative-type hair dye formulations (Ames et al., 1975). It may also be contained in indelible ink, antifreeze, and nail polish. Production statistics for 2,5-toluenediamine or its sulfate salt are not available; however, U.S.

*The CAS registry number is 6369-59-1.

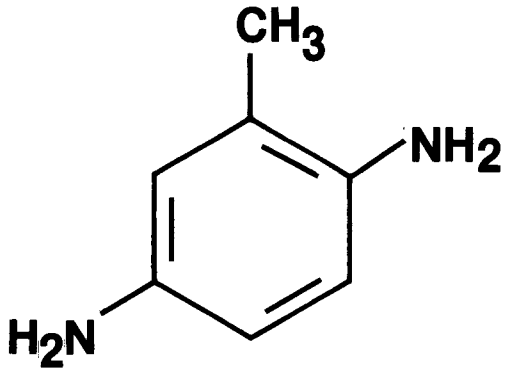

FIGURE 1
CHEMICAL STRUCTURE OF 2,5-TOLUENEDIAMINE (SULFATE)

imports of this chemical through principal U.S. customs districts amounted to 64,680 pounds in 1974 (U.S. International Trade Commission, 1976).

The potential for exposure to 2,5-toluenediamine is greatest among workers in the dye manufacturing industry, although fur dyers and manufacturers of inks as well as printers, engravers and lithographers may also experience significant daily contact with the chemical. Epidemiological studies suggest a relationship between occupational exposure to printing ink and increased incidence of cancer of both the bladder and the liver (Hoover and Fraumeni, 1975).

Exposure to 2,5-toluenediamine is widespread among the general population due to the increasingly common practice of hair dyeing. It has been estimated that approximately 33 million women in the United States dye their hair, often monthly over a period of many years (Staats, 1977).

2,5-Toluenediamine is toxic following both ingestion and inhalation (Sax, 1975). It can also be absorbed through the skin. Three hours after application of 2,5-toluenediamine to the abdomen of dogs, skin penetration was indicated by blood and urine measurements (Kiese et al., 1968). When applied to the scalp of human volunteers in conjunction with detergents, resorcinol and hydrogen peroxide, the diacetyl derivative was excreted in the urine for two days following application, indicating absorption and metabolism through this route as well (Kiese and Rauscher, 1968). The compound has a toxic action

upon the liver and can cause fatty degeneration of that organ. In addition, it is toxic to the central nervous system and produces anemia by destruction of red blood cells. The compound is considered to be highly irritating, producing irritation and blisters on the fingers of sensitive individuals; permanent injury to an eye was reported following use of 2,5-toluenediamine as an eyelash dye (Sax, 1975).

In studies by Ames et al. (1975), 2,5-toluenediamine was found to cause frameshift mutations in a tester strain (TA 1538) of *Salmonella typhimurium* in the presence of rat liver microsomes. Oxidation of 2,5-toluenediamine by hydrogen peroxide prior to testing resulted in a fortyfold increase in mutagenic activity.

II. MATERIALS AND METHODS

A. Chemicals

2,5-Toluenediamine sulfate was purchased from the Wayland Chemical Division of the Philip A. Hunt Chemical Corporation, Lincoln, Rhode Island. Analysis by the manufacturer suggested a purity of 99 percent with 25 ppm iron, 0.6 percent volatiles, and a maximum of 0.1 percent moisture. Analysis was also performed by Mason Research Institute, Worcester, Massachusetts. The melting point determination showed decomposition above 275°C. No literature value was found for comparison. Thin-layer chromatography utilizing two solvent systems, hexane:benzene:methanol and hexane:ethyl acetate, did not indicate the presence of any impurities. The evidence suggested a compound of high purity.

Throughout this report the term 2,5-toluenediamine sulfate is used to represent this material.

B. Dietary Preparation

The basal laboratory diet for both dosed and control animals consisted of Wayne Lab-Blox® (Allied Mills, Inc., Chicago, Illinois). 2,5-Toluenediamine sulfate was administered to the dosed animals as a component of the diet. The chemical was mixed with the feed in a 6 kg capacity Patterson-Kelley standard model stainless steel twin-shell V-blender. After 20 minutes of blending, the mixtures were placed in double plastic bags and stored in the dark at 4°C. Mixtures were prepared weekly and stored for not longer than 2 weeks.

C. Animals

Two animal species, rats and mice, were used in the chronic carcinogenicity bioassay. Fischer 344 rats and B6C3F1 mice were obtained through contracts of the Division of Cancer Treatment, National Cancer Institute. All mice and the high dose rats and their controls were supplied by Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts. The low dose rats and their controls were supplied by Laboratory Supply Co., Inc., Indianapolis, Indiana. Except for the low dose rats and their controls, all the other dosed animals were received in separate shipments from their respective controls.

Upon arrival, a sample of animals was examined for parasites and other signs of disease. The remaining animals were quarantined by species for 2 weeks prior to initiation of test. Animals were assigned to groups and distributed among cages so that the average body weight per cage was approximately equal for a given sex and species.

D. Animal Maintenance

All animals were housed by species in rooms having a temperature range of 23° to 34°C. Incoming air was filtered through Tri-Dek® 15/40 denier Dacron® filters (Tri-Dim Filter Corp., Hawthorne, New Jersey) providing six changes of room air per hour. Fluorescent lighting was provided on a 12-hour-daily cycle.

Rats were housed five per cage by sex. During quarantine and for the first 13 months of study, low dose rats and their controls

were kept in galvanized- or stainless-steel wire-mesh cages suspended above newspapers. High dose rats and their controls were housed in galvanized-steel wire-mesh cages during quarantine and for the first 11 months of study. Newspapers under cages were replaced daily and cages and racks washed weekly. For the remainder of the study, all rats were housed in suspended polycarbonate cages equipped with disposable nonwoven filter sheets. Clean bedding and cages were provided twice weekly. Low dose rats and their controls received Bed-o-Cobs® corncob bedding (The Andersons Cob Division, Maumee, Ohio) for the first 8 months that they were housed in polycarbonate cages. Thereafter, SAN-I-CEL® corncob bedding (Paxton Processing Company, Paxton, Illinois) was used. High dose rats and their controls were provided with SAN-I-CEL® for the first 11 months that they were housed in polycarbonate cages, and then Aspen hardwood chip bedding (American Excelsior Company, Baltimore, Maryland) was provided for the remainder of the study. Stainless steel cage racks were cleaned once every 2 weeks and disposable filters were replaced at that time.

Mice were housed by sex in polycarbonate cages. During quarantine and periods of chemical administration, cages were fitted with perforated stainless steel lids. Stainless steel wire bar lids were used during the final observation period. Both types of lids were supplied by Lab Products, Inc., Garfield, New Jersey. Nonwoven fiber filter bonnets were used over cage lids. Low dose mice and their

controls were housed ten per cage for the first 18 months of study and five per cage thereafter. The number of high dose mice per cage was reduced from ten to five after 13 months. The number of high dose controls per cage was reduced to five after 12 months. Clean cages, lids, filters, and bedding were provided three times per week when cage populations were ten and twice per week when cage populations were reduced to five. Bedding was the same as that provided to rats. Ab-sorb-dri® (Wilner Wood Products Company, Norway, Maine) hardwood chips were supplied for 2 months to the high dose mice and their controls and for 8 months to the low dose mice and their controls. Both groups received SAN-I-CEL® for the next 12 months. Bed-o-Cobs® was used until the end of the study. Reusable filter bonnets and pipe racks were sanitized every 2 weeks throughout the study.

Water was available to both species from 250 ml water bottles equipped with rubber stoppers and stainless steel sipper tubes. Bottles were replaced twice weekly and, for rats only, refilled as needed between changes.

Pelleted Wayne Lab-Blox® was supplied to all animals during the initial quarantine and final observation periods. During the dosing period, all animals were fed Wayne Lab-Blox® meal containing the appropriate concentration of 2,5-toluenediamine sulfate. Control animals had untreated meal available *ad libitum*. Meal was supplied throughout the study to all mice and to low dose rats and their controls in Alpine® aluminum feed cups (Curtin Matheson Scientific, Inc.,

Woburn, Massachusetts) containing stainless steel baffles. High dose rats and their controls were fed from Alpine® feed cups for the first 14 months of study and from stainless steel gangstyle hoppers (Scientific Cages, Inc., Bryan, Texas) for the remainder of the study. During the final observation period, mice were fed pellets from a wire bar hopper incorporated into the cage lid, and rats were fed pellets on the cage floor. Food hoppers were changed on the same schedule as were cages. Food was replenished daily in Alpine® feed cups.

Dosed rats were housed in a room with other rats receiving diets containing* acetylaminofluorene (53-96-3); a mixture of dulcin (150-69-6) and L-arginine glutamate (4320-30-3); sodium nitrite (7632-00-0); L-arginine glutamate (4320-30-3); N-butylurea (592-31-4); 2-chloro-p-phenylenediamine sulfate (61702-44-1); N,N-dimethyl-p-nitrosoaniline (138-89-6); 2,4-dinitrotoluene (121-14-2); 4-nitroanthranilic acid (619-17-0); 1,5-naphthalenediamine (2243-62-1); N-(1-naphthyl)ethylenediamine dihydrochloride (1465-25-4); and aniline hydrochloride (142-04-1). Control rats were housed in a room with other rats receiving diets containing 1-nitronaphthalene (86-57-7); 5-nitro-o-toluidine (99-55-8); hydrazobenzene (530-50-7); 2-aminoanthraquinone (117-79-3); 6-nitrobenzimidazole (94-52-0); 3-amino-9-ethylcarbazole hydrochloride; 2,4-diaminoanisole sulfate (615-05-4); and APC (8003-03-0).

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*CAS registry numbers are given in parentheses.

High dose mice shared a room with other mice receiving diets containing 5-nitro-o-toluidine (99-55-8); hydrazobenzene (530-50-7); 3-amino-9-ethylcarbazole hydrochloride; 1-nitronaphthalene (86-57-7); 6-nitrobenzimidazole (94-52-0); 5-nitro-o-anisidine (99-59-2); and 2,4-diaminoanisole sulfate (615-05-4). High dose control mice were housed in a room with other mice receiving diets containing 2-methyl-1-nitroanthraquinone (129-15-7); 4-chloro-m-phenylenediamine (5131-60-2); acetylaminofluorene (53-96-3); p-cresidine (120-71-8); and fenaminosulf (140-56-7). Low dose mice and their controls were in a room with other mice receiving diets containing amitrole (61-82-5); APC (8003-03-0); N,N-dimethyl-p-nitrosoaniline (138-89-6); 2,4-dinitrotoluene (121-14-2); 4-nitroanthranilic acid (619-17-0); 2-aminoanthraquinone (117-79-3); 3-amino-4-ethoxyacetanilide (17026-81-2); 3-amino-9-ethylcarbazole hydrochloride; 1-amino-2-methylanthraquinone (82-28-0); 5-nitro-o-anisidine (99-59-2); 1-nitronaphthalene (86-57-7); 5-nitroacenaphthene (602-87-9); 3-nitro-p-acetophenetide (1777-84-0); and 2,4-diaminoanisole sulfate (615-05-4).

E. Selection of Initial Concentrations

In order to establish the maximum tolerated concentrations of 2,5-toluenediamine sulfate for administration to dosed animals in the chronic studies, subchronic toxicity tests were conducted with both Fischer 344 rats and C57BL/6 mice.* Rats and mice were distributed among five groups, each consisting of five males and five

*This strain was used due to the unavailability at that time of B6C3F1 mice.

females. 2,5-Toluenediamine sulfate was incorporated into the basal laboratory diet and fed ad libitum to four of the five rat groups and four of the five mouse groups in concentrations of 0.02, 0.05, 0.08, and 0.11 percent. The remaining group of each species served as control groups, receiving only the basal laboratory diet. The dosed dietary preparations were administered for a period of 4 weeks, followed by 2 weeks of observation.

All animals survived until necropsy. Mean body weight depression relative to controls was observed in all dosed rat groups and in dosed female mice; however, the depression was not dose-related. No mean body weight depression was observed in male mice.

The high concentrations selected for administration in the chronic study were 0.05 percent for rats and 0.06 percent for mice.

F. Experimental Design

The experimental design parameters for the chronic study (species, sex, group size, actual concentrations administered, duration of treated and untreated observation periods, and time-weighted average concentrations) are summarized in Tables 1 and 2.

At initiation of the study, all animals were approximately 6 weeks old. High dose rats and their controls were placed on test 11 months after low dose rats and their controls. Each dosed rat group was placed on test during the same week as its respective control group. Rats initially received 2,5-toluenediamine sulfate at dietary concentrations of 0.05 and 0.03 percent. Because of a lack

TABLE 1

DESIGN SUMMARY FOR FISCHER 344 RATS
2,5-TOLUENEDIAMINE SULFATE FEEDING EXPERIMENT

	INITIAL GROUP SIZE	2,5-TOLUENEDIAMINE SULFATE CONCENTRATION (PERCENT)	OBSERVATION PERIOD TREATED (WEEKS)	OBSERVATION PERIOD UNTREATED (WEEKS)	TIME-WEIGHTED AVERAGE CONCENTRATION[a]
MALE					
LOW DOSE CONTROL	50	0	0	107	0
HIGH DOSE CONTROL	25	0	0	109	0
LOW DOSE	50	0.05 0.06 0	14 64	 28	0.06
HIGH DOSE	50	0.2 0	78	 30	0.2
FEMALE					
LOW DOSE CONTROL	50	0	0	108	0
HIGH DOSE CONTROL	25	0	0	109	0
LOW DOSE	50	0.05 0.06 0	14 64	 29	0.06
HIGH DOSE	50	0.2 0	78	 31	0.2

[a] Time-weighted average concentration = $\frac{\Sigma(\text{concentration X weeks received})}{\Sigma(\text{weeks receiving chemical})}$

TABLE 2

DESIGN SUMMARY FOR B6C3F1 MICE
2,5-TOLUENEDIAMINE SULFATE FEEDING EXPERIMENT

	INITIAL GROUP SIZE	2,5-TOLUENEDIAMINE SULFATE CONCENTRATION (PERCENT)	OBSERVATION PERIOD TREATED (WEEKS)	OBSERVATION PERIOD UNTREATED (WEEKS)
MALE				
LOW DOSE CONTROL	50	0	0	96
HIGH DOSE CONTROL	50	0	0	98
LOW DOSE	50	0.06	78	
		0		16
HIGH DOSE	50	0.1	78	
		0		19
FEMALE				
LOW DOSE CONTROL	50	0	0	97
HIGH DOSE CONTROL	50	0	0	98
LOW DOSE	50	0.06	78	
		0		16
HIGH DOSE	50	0.1	78	
		0		19

of observed mean body weight depression or other clinical signs at a level of 0.05 percent, the groups receiving 0.03 percent were terminated 2 months after initiation, and a new group was initiated at a concentration of 0.2 percent along with a new control group. Throughout this report those rats receiving a concentration of 0.2 percent and their controls are referred to as the high dose and high dose control groups, respectively, while those rats initially receiving a concentration of 0.05 percent and their controls are referred to as the low dose and low dose control groups, respectively. From week 15, the concentration received by the low dose group was increased from 0.05 to 0.06 percent.

Low dose mice were placed on test 6 months before high dose mice. Low dose mice were placed on test 2 weeks before their controls. High dose mice were placed on test 2 months after their controls. The initial dietary concentrations utilized for mice were 0.06 and 0.03 percent. Again, because of a lack of observed mean body weight depression or other clinical signs, the group receiving 0.03 percent was discontinued 6 months after initiation and a new group was initiated at a concentration of 0.1 percent, accompanied by a new control group. Throughout this report those mice receiving a concentration of 0.1 percent and their controls are referred to as the high dose and high dose control groups, respectively, while those mice receiving a concentration of 0.06 percent and their controls are referred to as the low dose and low dose control groups, respectively.

G. Clinical and Histopathologic Examinations

Animals were weighed immediately prior to initiation of the experiment. Body weights were recorded twice weekly for the first 12 weeks of the study and at monthly intervals thereafter. Food consumption, for two cages from each group, was monitored for seven consecutive days once a month for the first nine months of the bioassay and for three consecutive days each month thereafter. The presence of tissue masses and lesions was determined by monthly observation and palpation of each animal. From the first day, all animals were inspected twice daily for mortality.

A necropsy was performed on each animal regardless of whether it died, was killed when moribund, or was sacrificed at the end of the bioassay. The animals were euthanized by carbon dioxide inhalation, and were immediately necropsied. The histopathologic examination consisted of gross and microscopic examination of major tissues, organs, and gross lesions taken from sacrificed animals and, whenever possible, from animals found dead.

Tissues were preserved in 10 percent buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin prior to microscopic examination. An occasional section was subjected to special staining techniques for more definitive diagnosis.

Slides were prepared from the following tissues: skin, subcutaneous tissue, lungs and bronchi, trachea, bone marrow, spleen, lymph

nodes, thymus, heart, salivary gland, liver, gallbladder (mice), pancreas, esophagus, stomach, small intestine, large intestine, kidney, urinary bladder, pituitary, adrenal, thyroid, parathyroid, testis, prostate, seminal vesicle, brain, tunica vaginalis, muscle, ear, uterus, mammary gland, and ovary.

A few tissues were not examined for some animals, particularly for those that died early. Also, some animals were missing, cannibalized, or judged to be in such an advanced state of autolysis as to preclude histopathologic interpretation. Thus, the number of animals for which particular organs, tissues, or lesions were examined microscopically varies and does not necessarily represent the number of animals that were placed on experiment in each group.

H. Data Recording and Statistical Analyses

Pertinent data on this experiment have been recorded in an automatic data processing system, the Carcinogenesis Bioassay Data System (Linhart et al., 1974). The data elements include descriptive information on the chemicals, animals, experimental design, clinical observations, survival, body weight, and individual pathologic results, as recommended by the International Union Against Cancer (Berenblum, 1969). Data tables were generated for verification of data transcription and for statistical review.

These data were analyzed using the statistical techniques described in this section. Those analyses of the experimental results that bear on the possibility of carcinogenicity are discussed in the statistical narrative sections.

Probabilities of survival were estimated by the product-limit procedure of Kaplan and Meier (1958) and are presented in this report in the form of graphs. Animals were statistically censored as of the time that they died of other than natural causes or were found to be missing; animals dying from natural causes were not statistically censored. Statistical analyses for a possible dose-related effect on survival used the method of Cox (1972) when testing two groups for equality and used Tarone's (1975) extensions of Cox's methods when testing a dose-related trend. One-tailed P-values have been reported for all tests except the departure from linearity test, which is only reported when its two-tailed P-value is less than 0.05.

The incidence of neoplastic or nonneoplastic lesions has been given as the ratio of the number of animals bearing such lesions at a specific anatomic site (numerator) to the number of animals in which that site was examined (denominator). In most instances, the denominators included only those animals for which that site was examined histologically. However, when macroscopic examination was required to detect lesions prior to histologic sampling (e.g., skin or mammary tumors), or when lesions could have appeared at multiple sites (e.g., lymphomas), the denominators consist of the numbers of animals necropsied.

The purpose of the statistical analyses of tumor incidence is to determine whether animals receiving the test chemical developed a significantly higher proportion of tumors than did the control animals.

As a part of these analyses, the one-tailed Fisher exact test (Cox, 1970, pp. 48-52) was used to compare the tumor incidence of a control group to that of a group of treated animals at each dose level. When results for a number of treated groups, k, are compared simultaneously with those for a control group, a correction to ensure an overall significance level of 0.05 may be made. The Bonferroni inequality (Miller, 1966, pp. 6-10) requires that the P-value for any comparison be less than or equal to 0.05/k. In cases where this correction was used, it is discussed in the narrative section. It is not, however, presented in the tables, where the Fisher exact P-values are shown.

The Cochran-Armitage test for linear trend in proportions, with continuity correction (Armitage, 1971, pp. 362-365), was also used when appropriate. Under the assumption of a linear trend, this test determined if the slope of the dose-response curve is different from zero at the one-tailed 0.05 level of significance. Unless otherwise noted, the direction of the significant trend was a positive dose relationship. This method also provides a two-tailed test of departure from linear trend.

A time-adjusted analysis was applied when numerous early deaths resulted from causes that were not associated with the formation of tumors. In this analysis, deaths that occurred before the first tumor was observed were excluded by basing the statistical tests on animals that survived at least 52 weeks, unless a tumor was found at the anatomic site of interest before week 52. When such an early

tumor was found, comparisons were based exclusively on animals that survived at least as long as the animal in which the first tumor was found. Once this reduced set of data was obtained, the standard procedures for analyses of the incidence of tumors (Fisher exact tests, Cochran-Armitage tests, etc.) were followed.

When appropriate, life-table methods were used to analyze the incidence of tumors. Curves of the proportions surviving without an observed tumor were computed as in Saffiotti et al. (1972). The week during which animals died naturally or were sacrificed was entered as the time point of tumor observation. Cox's methods of comparing these curves were used for two groups; Tarone's extension to testing for linear trend was used for three groups. The statistical tests for the incidence of tumors which used life-table methods were one-tailed and, unless otherwise noted, in the direction of a positive dose relationship. Significant departures from linearity ($P < 0.05$, two-tailed test) were also noted.

The approximate 95 percent confidence interval for the relative risk of each dosed group compared to its control was calculated from the exact interval on the odds ratio (Gart, 1971). The relative risk is defined as p_t/p_c where p_t is the true binomial probability of the incidence of a specific type of tumor in a treated group of animals and p_c is the true probability of the spontaneous incidence of the same type of tumor in a control group. The hypothesis of equality between the true proportion of a specific tumor in a treated group

and the proportion in a control group corresponds to a relative risk of unity. Values in excess of unity represent the condition of a larger proportion in the treated group than in the control.

The lower and upper limits of the confidence interval of the relative risk have been included in the tables of statistical analyses. The interpretation of the limits is that in approximately 95 percent of a large number of identical experiments, the true ratio of the risk in a treated group of animals to that in a control group would be within the interval calculated from the experiment. When the lower limit of the confidence interval is greater than one, it can be inferred that a statistically significant result (a $P < 0.025$ one-tailed test when the control incidence is not zero, $P < 0.050$ when the control incidence is zero) has occurred. When the lower limit is less than unity but the upper limit is greater than unity, the lower limit indicates the absence of a significant result while the upper limit indicates that there is a theoretical possibility of the induction of tumors by the test chemical which could not be detected under the conditions of this test.

III. CHRONIC TESTING RESULTS: RATS

A. Body Weights and Clinical Observations

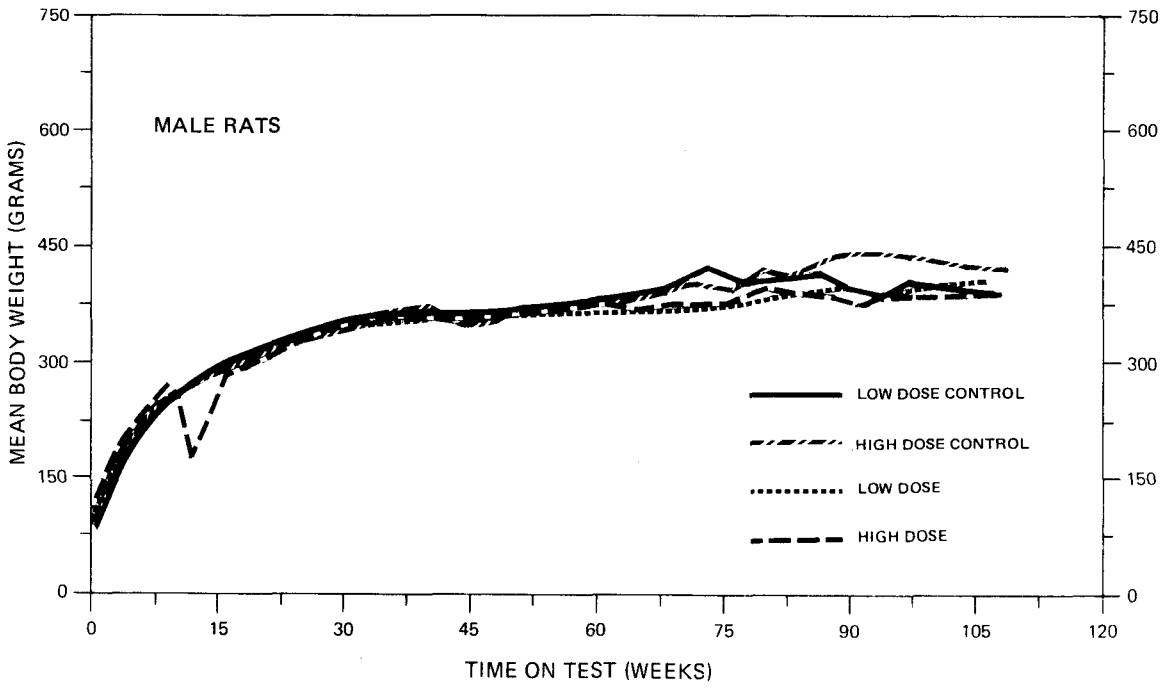
When compared to their respective controls, the high dose female rats exhibited consistent mean body weight depression. This trend was not as evident in the other groups of dosed rats (Figure 2).

Only isolated clinical observations were reported. These included crusted lesions on the dorsolateral surface in one low dose control male and one low dose male and on the side of the head in one low dose female; tissue masses on the ear in two low dose males and on the foreleg in one low dose male; a subcutaneous mass under the base of the tail in one high dose male; and growths on the foreleg in three low dose females and on the ear in another low dose female.

B. Survival

The estimated probabilities of survival for male and female rats in the control and 2,5-toluenediamine sulfate-dosed groups are shown in Figure 3. For both male and female rats there was no significant association between dosage and mortality.

There were adequate numbers of male rats at risk from late-developing tumors despite the sacrifice in week 78 of five rats from the low dose and each control group, and the sacrifice in week 29 of ten additional rats from the low dose control group. Five males from the high dose group died in week 78. Ninety percent (45/50) of the high dose, 84 percent (42/50) of the low dose, 72 percent (18/25) of



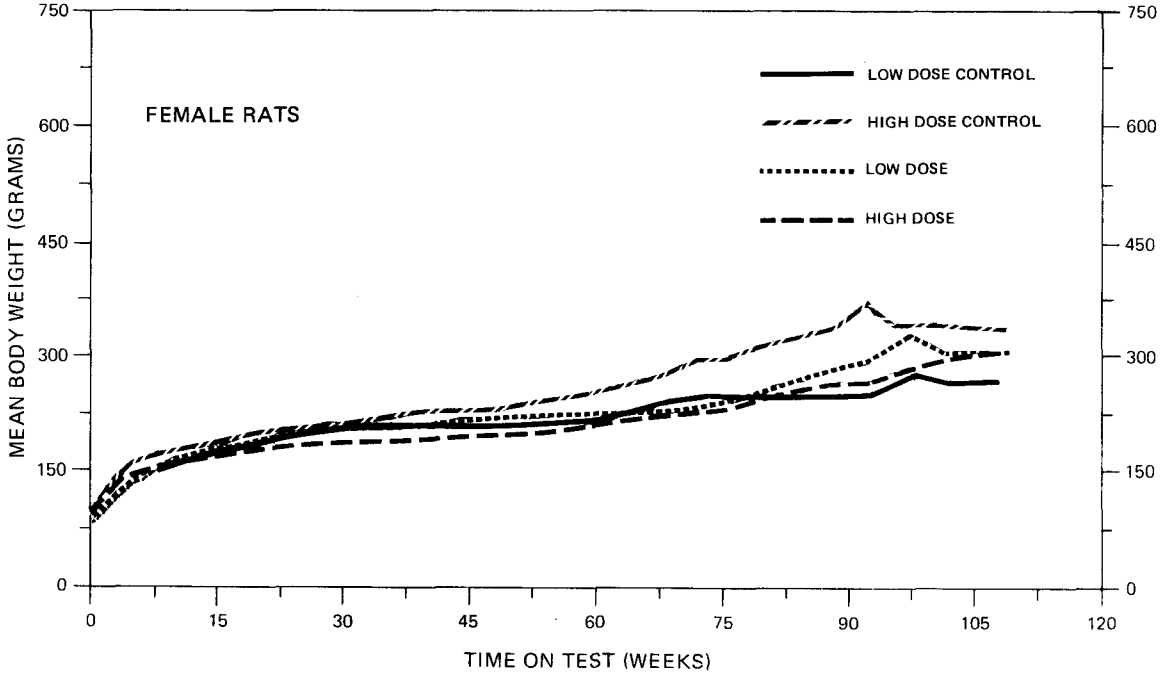
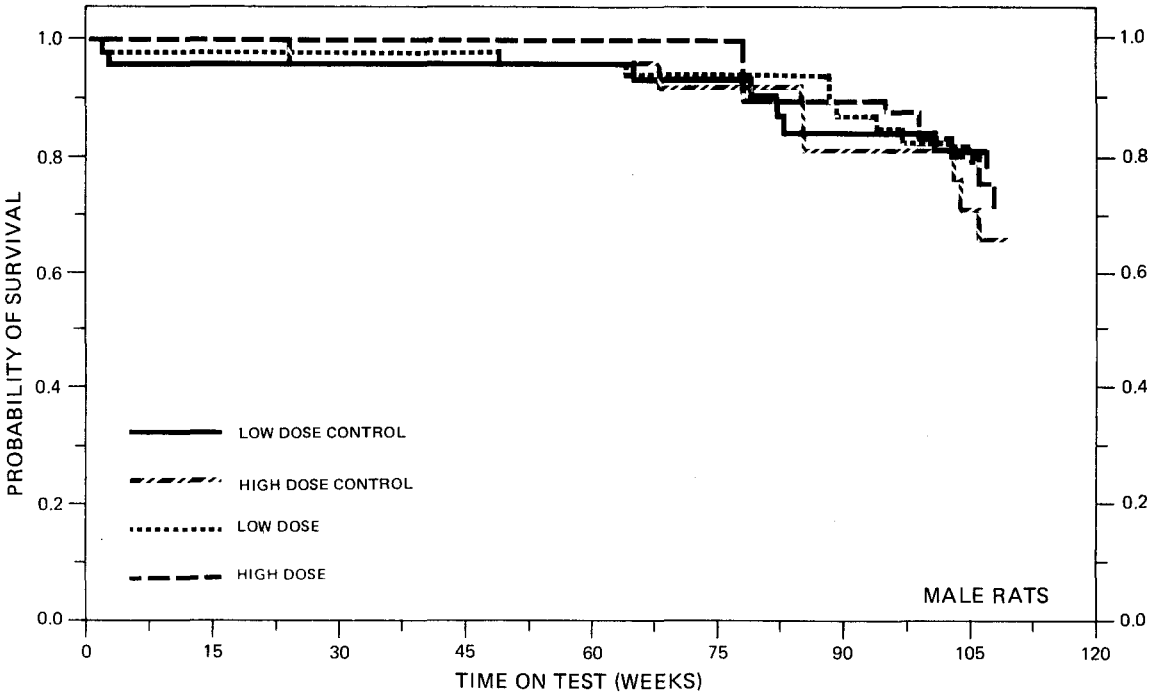


FIGURE 2
GROWTH CURVES FOR 2,5-TOLUENEDIAMINE SULFATE CHRONIC STUDY RATS



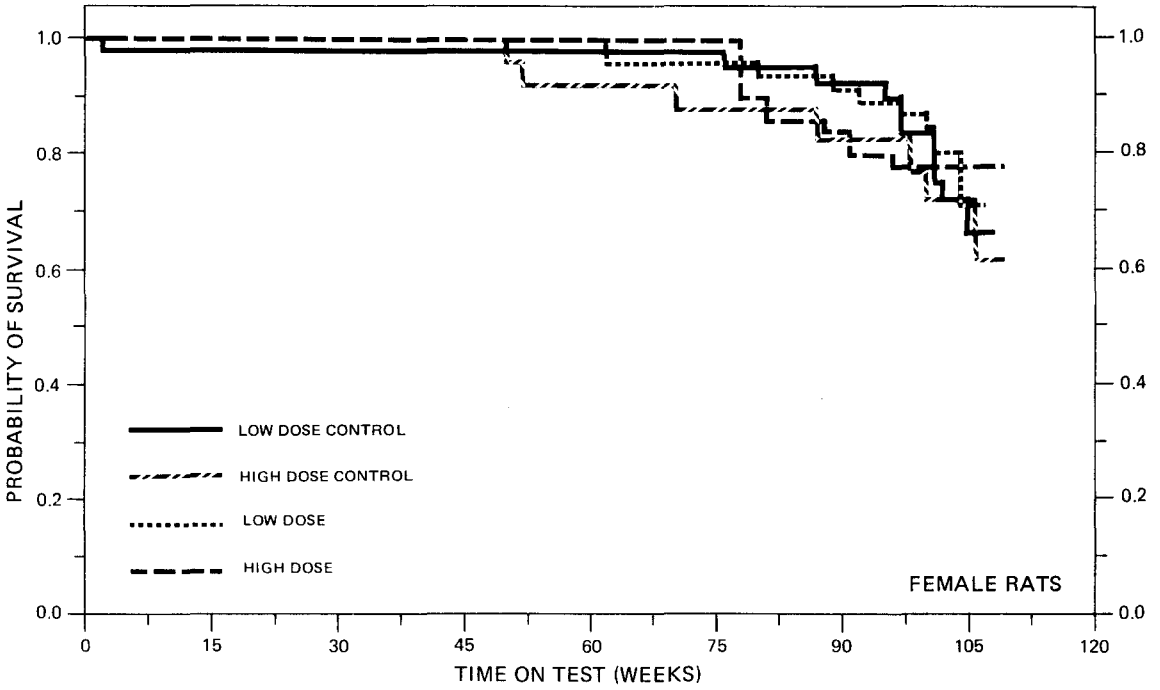


FIGURE 3
SURVIVAL COMPARISONS OF 2,5-TOLUENEDIAMINE SULFATE CHRONIC STUDY RATS

the high dose control, and 58 percent (29/50) of the low dose control group survived on test at least 85 weeks.

There were adequate numbers of female rats at risk from late-developing tumors despite the sacrifice in week 78 of five rats from the low dose and each control group and the sacrifice in week 29 of ten additional rats from the low dose control group. Five of the high dose females died in week 78. Eighty-six percent (43/50) of the high dose, 84 percent (42/50) of the low dose, 68 percent (17/25) of the high dose control, and 66 percent (33/50) of the low dose control group survived on test at least 85 weeks.

C. Pathology

Histopathologic findings on neoplasms in rats are summarized in Appendix A (Tables A1 and A2); findings on nonneoplastic lesions are summarized in Appendix C (Tables C1 and C2).

A variety of neoplasms which are routinely seen in this strain of rat was recorded. The incidences of these tumors in dosed rats were not judged to be attributable to the administration of 2,5-toluenediamine sulfate.

Nonneoplastic lesions which commonly occur in aging Fischer 344 rats and which were unrelated to the administration of the test compound were seen.

This pathologic examination provided no evidence for the carcinogenicity of 2,5-toluenediamine sulfate in Fischer 344 rats.

D. Statistical Analyses of Results

The results of the statistical analyses of tumor incidence in rats are summarized in Tables 3 and 4. The analysis for every type of tumor that was observed in more than 5 percent of any of the 2,5-toluenediamine sulfate-dosed groups of either sex is included. In addition to these analyses, time-adjusted analyses were performed based upon animals surviving at least 52 weeks; the time-adjusted analyses did not produce different conclusions from the analyses presented here.

In male rats, the Fisher exact test indicated a significant incidence of interstitial-cell tumors of the testis when the high dose group was compared to the high dose control ($P = 0.014$). The comparison of low dose to low dose control had a probability level of $P = 0.039$, which was not significant under the Bonferroni criterion. These results were discounted, however, due to the well-known high variation in the spontaneous incidence of this tumor (Cockrell and Garner, 1976).

For females the Fisher exact test indicated a significantly ($P = 0.006$) lower incidence of pituitary adenomas in the low dose group than in the low dose control group. The high dose comparison was not significant.

No other test for any site in either male or female rats was significant under the Bonferroni criterion. Based upon these statistical results there was no convincing evidence of the carcinogenicity of 2,5-toluenediamine sulfate in rats.

TABLE 3

ANALYSES OF THE INCIDENCE OF PRIMARY TUMORS AT SPECIFIC SITES IN MALE RATS TREATED WITH 2,5-TOLUENEDIAMINE SULFATE[a]

TOPOGRAPHY:MORPHOLOGY	LOW DOSE CONTROL	HIGH DOSE CONTROL	LOW DOSE	HIGH DOSE
Lung: Alveolar/Bronchiolar Adenoma or Alveolar/Bronchiolar Carcinoma[b]	0/46(0.00)	3/25(0.12)	1/48(0.02)	0/49(0.00)
P Values[c]	---	---	N.S.	P = 0.035(N)
Relative Risk (Control)[d]	---	---	Infinite	0.000
Lower Limit	---	---	0.051	0.000
Upper Limit	---	---	Infinite	0.843
Weeks to First Observed Tumor	---	78	97	---
Hematopoietic System: Leukemia or Malignant Lymphoma[b]	2/46(0.04)	4/25(0.16)	5/49(0.10)	8/49(0.16)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	2.347	1.020
Lower Limit	---	---	0.407	0.310
Upper Limit	---	---	23.709	4.278
Weeks to First Observed Tumor	79	85	89	99
Pituitary: Adenoma NOS, Basophil Adenoma or Chromophobe Adenoma[b]	12/41(0.29)	3/21(0.14)	3/45(0.07)	3/40(0.08)
P Values[c]	---	---	P = 0.006(N)	N.S.
Relative Risk (Control)[d]	---	---	0.228	0.525
Lower Limit	---	---	0.044	0.078
Upper Limit	---	---	0.772	3.650
Weeks to First Observed Tumor	101	78	106	108

TABLE 3 (CONTINUED)

TOPOGRAPHY:MORPHOLOGY	LOW DOSE CONTROL	HIGH DOSE CONTROL	LOW DOSE	HIGH DOSE
Adrenal: Pheochromocytoma or Pheochromocytoma Malignant[b]	6/43(0.14)	4/25(0.16)	2/48(0.04)	1/48(0.02)
P Values[c]	---	---	N.S.	P = 0.044(N)
Relative Risk (Control)[d]	---	---	0.299	0.130
Lower Limit	---	---	0.031	0.003
Upper Limit	---	---	1.568	1.237
Weeks to First Observed Tumor	107	68	103	108
Thyroid: Papillary Adenocarcinoma[b]	0/45(0.00)	0/23(0.00)	3/46(0.07)	0/47(0.00)
P Values[c]	---	---	N.S.	---
Relative Risk (Control)[d]	---	---	Infinite	---
Lower Limit	---	---	0.590	---
Upper Limit	---	---	Infinite	---
Weeks to First Observed Tumor	---	---	106	---
Thyroid: Adenocarcinoma NOS, Papillary Adenocarcinoma, Cyst-adenocarcinoma NOS, or Papillary Cystadenocarcinoma NOS[b]	2/45(0.04)	0/23(0.00)	3/46(0.07)	3/47(0.06)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	1.467	Infinite
Lower Limit	---	---	0.176	0.303
Upper Limit	---	---	16.894	Infinite
Weeks to First Observed Tumor	107	---	106	105

TABLE 3 (CONTINUED)

TOPOGRAPHY:MORPHOLOGY	LOW DOSE CONTROL	HIGH DOSE CONTROL	LOW DOSE	HIGH DOSE
Pancreatic Islets: Islet-Cell Adenoma[b]	2/42(0.05)	2/25(0.08)	4/48(0.08)	1/48(0.02)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	1.750	0.260
Lower Limit	---	---	0.266	0.005
Upper Limit	---	---	18.600	4.803
Weeks to First Observed Tumor	107	109	106	108
Preputial Gland: Adenoma NOS or Carcinoma NOS[b]	0/46(0.00)	2/25(0.08)	0/49(0.00)	2/49(0.04)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	---	0.510
Lower Limit	---	---	---	0.040
Upper Limit	---	---	---	6.750
Weeks to First Observed Tumor	---	85	---	108
Testis: Interstitial-Cell Tumor[b]	33/45(0.73)	19/24(0.79)	43/48(0.90)	47/48(0.98)
P Values[c]	---	---	P = 0.039	P = 0.014
Relative Risk (Control)[d]	---	---	1.222	1.237
Lower Limit	---	---	0.981	1.017
Upper Limit	---	---	1.446	1.331
Weeks to First Observed Tumor	78	78	78	78

TABLE 3 (CONCLUDED)

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[a]Treated groups received time-weighted average doses of 0.06 or 0.20 percent in feed.

[b]Number of tumor-bearing animals/number of animals examined at site (proportion).

[c]The probability level for the Fisher exact test for the comparison of a treated group with its control group is given beneath the incidence of tumors in the treated group when $P < 0.05$; otherwise, not significant (N.S.) is indicated. A negative designation (N) indicates a lower incidence in the treated group than in the control group.

[d]The 95% confidence interval on the relative risk of the treated group to the control group.

TABLE 4

ANALYSES OF THE INCIDENCE OF PRIMARY TUMORS AT SPECIFIC SITES IN FEMALE RATS TREATED WITH 2,5-TOLUENEDIAMINE SULFATE[a]

TOPOGRAPHY:MORPHOLOGY	LOW DOSE CONTROL	HIGH DOSE CONTROL	LOW DOSE	HIGH DOSE
Hematopoietic System: Leukemia or Malignant Lymphoma[b]	4/49(0.08)	2/23(0.09)	2/50(0.04)	10/50(0.20)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	0.490	2.300
Lower Limit	---	---	0.046	0.553
Upper Limit	---	---	3.251	20.530
Weeks to First Observed Tumor	101	106	107	81
Pituitary: Adenoma NOS or Chromophobe Adenoma[b]	18/43(0.42)	8/21(0.38)	17/48(0.35)	10/43(0.23)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	0.846	0.611
Lower Limit	---	---	0.477	0.266
Upper Limit	---	---	1.507	1.546
Weeks to First Observed Tumor	76	78	80	108
Adrenal: Pheochromocytoma or Pheochromocytoma Malignant[b]	2/46(0.04)	3/23(0.13)	1/50(0.02)	1/48(0.02)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	0.460	0.160
Lower Limit	---	---	0.008	0.003
Upper Limit	---	---	8.542	1.882
Weeks to First Observed Tumor	108	109	107	109

TABLE 4 (CONTINUED)

TOPOGRAPHY:MORPHOLOGY	LOW DOSE CONTROL	HIGH DOSE CONTROL	LOW DOSE	HIGH DOSE
Thyroid: C-Cell Adenoma or C-Cell Carcinoma[b]	1/47(0.02)	3/21(0.14)	3/48(0.06)	0/49(0.00)
P Values[c]	---	---	N.S.	P = 0.025(N)
Relative Risk (Control)[d]	---	---	2.938	0.000
Lower Limit	---	---	0.246	0.000
Upper Limit	---	---	150.900	0.707
Weeks to First Observed Tumor	107	109	107	---
Mammary Gland: Adenocarcinoma NOS, Papillary Adenocarcinoma, Papillary Cystadenocarcinoma NOS or Infiltrating Duct Carcinoma[b]	2/49(0.04)	3/23(0.13)	1/50(0.02)	1/50(0.02)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	0.490	0.153
Lower Limit	---	---	0.008	0.003
Upper Limit	---	---	9.103	1.810
Weeks to First Observed Tumor	101	52	107	108
Mammary Gland: Fibroadenoma[b]	4/49(0.08)	4/23(0.17)	6/50(0.12)	9/50(0.18)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	1.470	1.035
Lower Limit	---	---	0.372	0.332
Upper Limit	---	---	6.681	4.234
Weeks to First Observed Tumor	101	109	100	108

TABLE 4 (CONCLUDED)

TOPOGRAPHY:MORPHOLOGY	LOW DOSE CONTROL	HIGH DOSE CONTROL	LOW DOSE	HIGH DOSE
Uterus: Endometrial Stromal Polyp[b]	10/48(0.21)	6/23(0.26)	7/49(0.14)	7/45(0.16)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	0.686	0.596
Lower Limit	---	---	0.241	0.199
Upper Limit	---	---	1.826	1.932
Weeks to First Observed Tumor	78	87	101	108
Uterus: Adenocarcinoma NOS[b]	4/48(0.08)	0/23(0.00)	3/49(0.06)	1/45(0.02)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	0.735	Infinite
Lower Limit	---	---	0.113	0.028
Upper Limit	---	---	4.114	Infinite
Weeks to First Observed Tumor	95	---	92	109

[a]Treated groups received time-weighted average doses of 0.06 or 0.20 percent in feed.

[b]Number of tumor-bearing animals/number of animals examined at site (proportion).

[c]The probability level for the Fisher exact test for the comparison of a treated group with its control group is given beneath the incidence of tumors in the treated group when $P < 0.05$; otherwise, not significant (N.S.) is indicated. A negative designation (N) indicates a lower incidence in the treated group than in the control group.

[d]The 95% confidence interval on the relative risk of the treated group to the control group.

To provide additional insight into the possible carcinogenicity of this compound, 95 percent confidence intervals on the relative risk have been estimated and entered in the tables based upon the observed tumor incidence rates. In many of the intervals shown in Tables 3 and 4, the value one is included; this indicates the absence of statistically significant results. It should also be noted that many of the confidence intervals have an upper limit greater than one, indicating the theoretical possibility of tumor induction in rats by 2,5-toluenediamine sulfate that could not be established under the conditions of this test.

IV. CHRONIC TESTING RESULTS: MICE

A. Body Weights and Clinical Observations

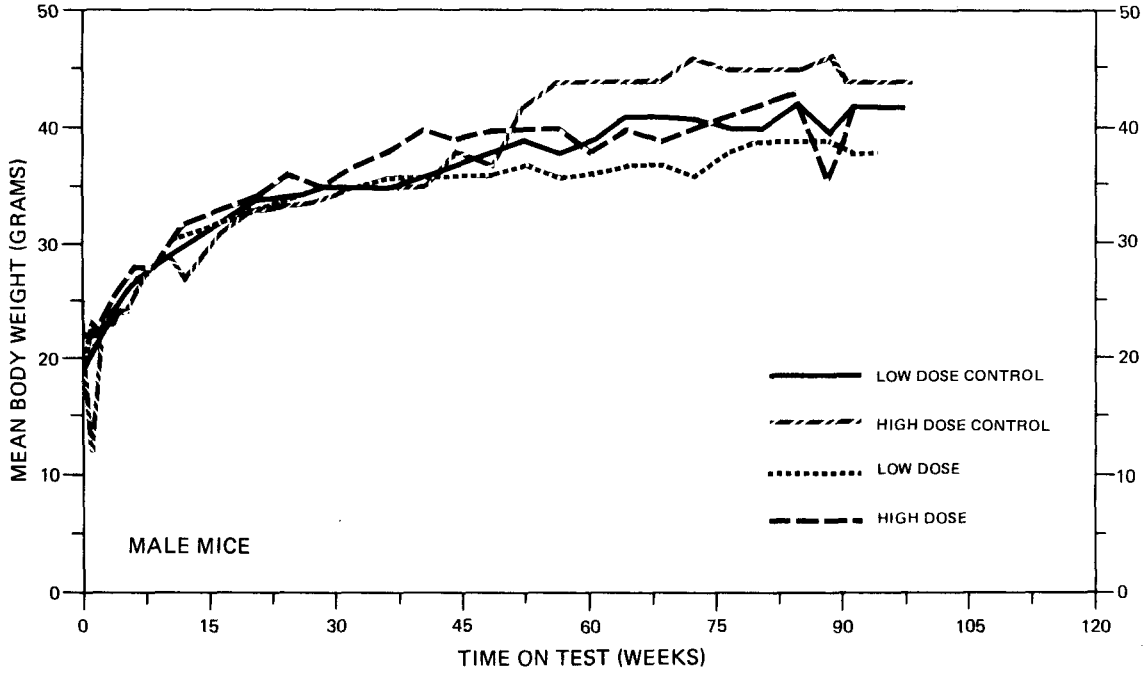
The only group exhibiting distinct compound-related mean body weight depression when compared to its control group was the high dose female group (Figure 4). It should be noted, however, that the growth pattern of the female high dose control group was unusual (i.e., it did not level off as the animals approached maturity).

No clinical abnormalities were recorded for dosed or control mice of either sex.

B. Survival

The estimated probabilities of survival for male and female mice in the control and 2,5-toluenediamine sulfate-dosed groups are shown in Figure 5. There was no significant association between dosage and mortality for male or female mice.

Five male and five female mice were sacrificed in week 78 from each group except the low dose group. There were adequate numbers of male mice at risk from late-developing tumors as 74 percent (37/50) of the high dose, 94 percent (47/50) of the low dose, 74 percent (37/50) of the high dose control, and 84 percent (42/50) of the low dose control mice survived on test until the end of the study. There were also adequate numbers of female mice at risk from late-developing tumors as 66 percent (33/50) of the high dose, 78 percent (39/50) of the low dose, 70 percent (35/50) of the high dose control, and 74



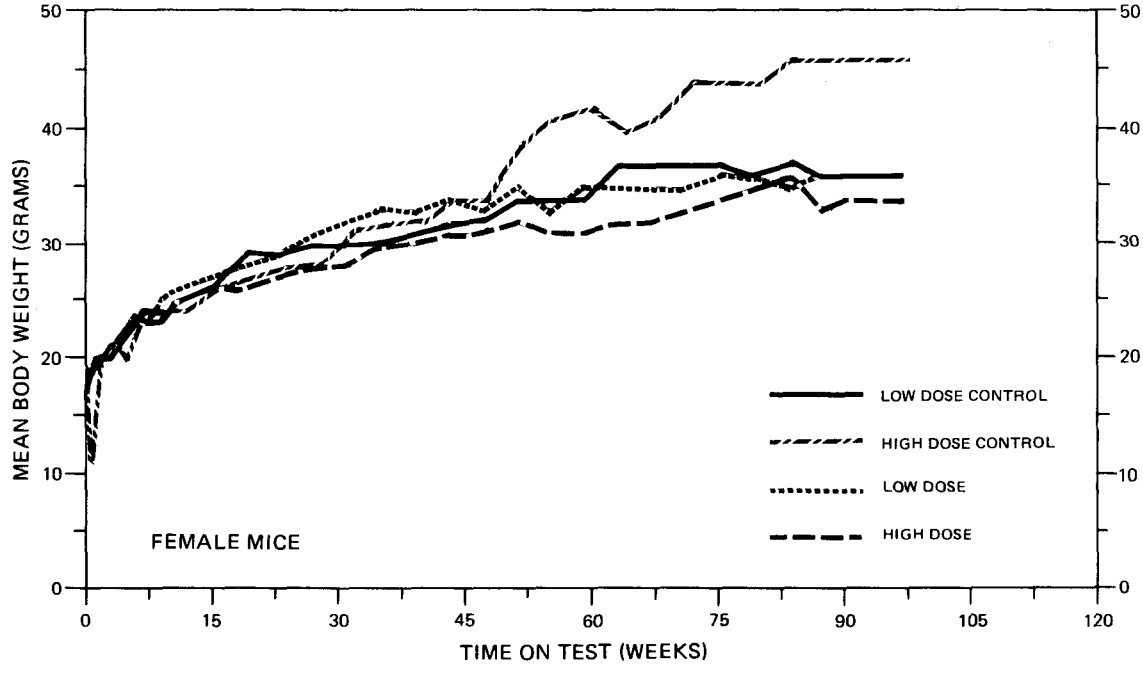
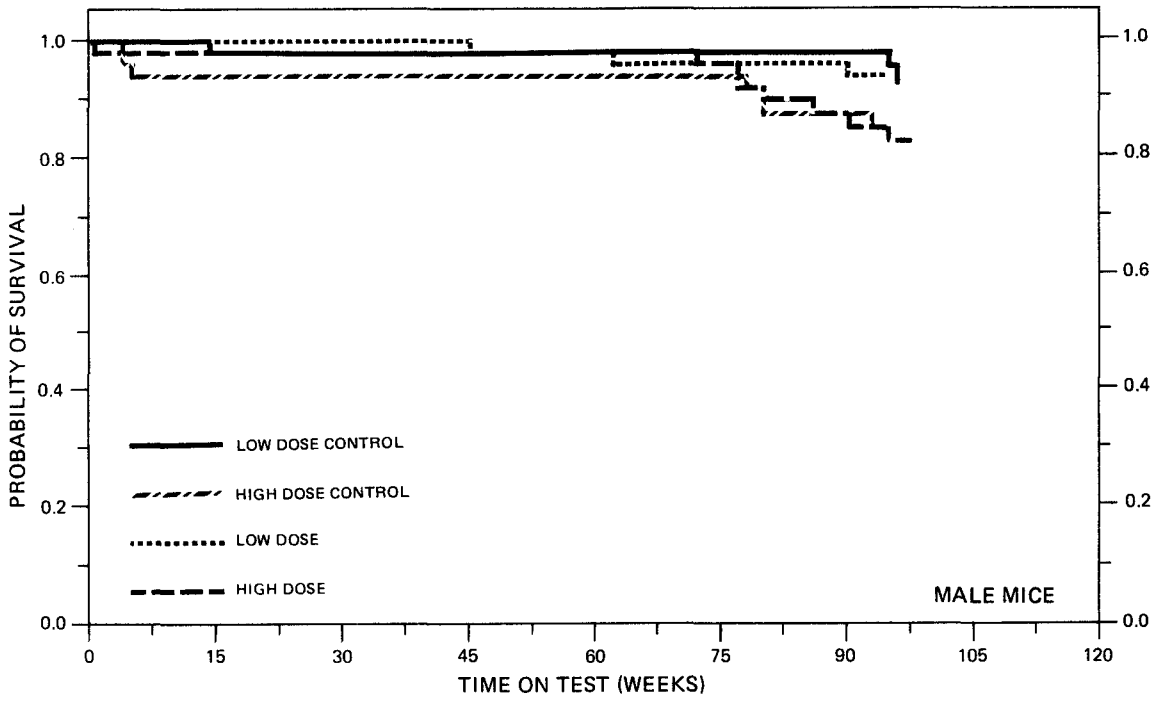


FIGURE 4
GROWTH CURVES FOR 2,5-TOLUENEDIAMINE SULFATE CHRONIC STUDY MICE



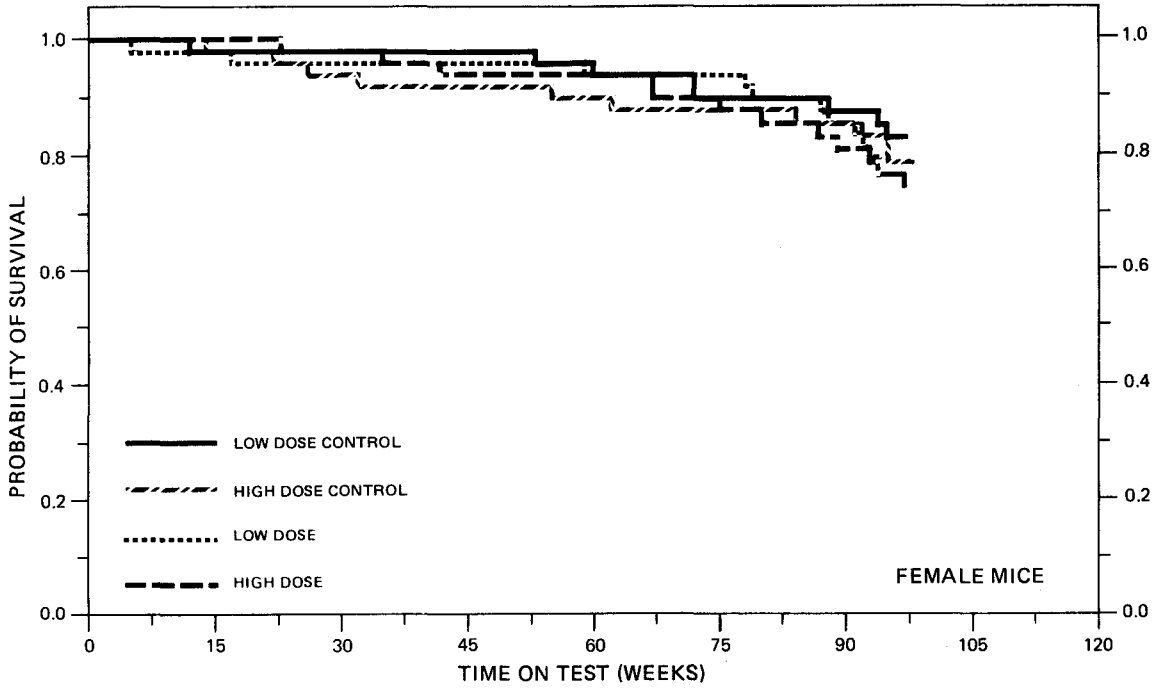


FIGURE 5
SURVIVAL COMPARISONS OF 2,5-TOLUENEDIAMINE SULFATE CHRONIC STUDY MICE

percent (37/50) of the low dose control mice survived on test until the end of the study.

C. Pathology

Histopathologic findings on neoplasms in mice are summarized in Appendix B (Tables B1 and B2); findings on nonneoplastic lesions are summarized in Appendix D (Tables D1 and D2).

Hepatocellular carcinoma occurred in 7/48 (15 percent) of the low dose control males, 10/45 (22 percent) of the high dose control males, 8/48 (17 percent) of the low dose males, and 16/49 (33 percent) of the high dose males. None of the tumors was judged to be due to the administration of the test chemical.

A variety of inflammatory and degenerative lesions which commonly occur in aging mice of this strain was seen. These nonneoplastic lesions were not considered to be compound-related.

This pathologic examination provided no evidence for the carcinogenicity of 2,5-toluenediamine sulfate in B6C3F1 mice.

D. Statistical Analyses of Results

The results of the statistical analyses of tumor incidence in mice are summarized in Tables 5 and 6. The analysis for every type of tumor that was observed in more than 5 percent of any of the 2,5-toluenediamine sulfate-dosed groups of either sex is included.

Elevated incidences of alveolar/bronchiolar adenomas and alveolar/bronchiolar carcinomas were observed among dosed female mice. For

TABLE 5

ANALYSES OF THE INCIDENCE OF PRIMARY TUMORS AT SPECIFIC SITES IN MALE MICE TREATED WITH 2,5-TOLUENEDIAMINE SULFATE[a]

TOPOGRAPHY:MORPHOLOGY	LOW DOSE CONTROL	HIGH DOSE CONTROL	LOW DOSE	HIGH DOSE
Lung: Alveolar/Bronchiolar Carcinoma[b]	6/48(0.13)	4/45(0.09)	1/47(0.02)	5/49(0.10)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	0.170	1.148
Lower Limit	---	---	0.004	0.264
Upper Limit	---	---	1.326	5.453
Weeks to First Observed Tumor	96	97	94	96
Lung: Alveolar/Bronchiolar Carcinoma or Alveolar/Bronchiolar Adenoma[b]	6/48(0.13)	11/45(0.24)	6/47(0.13)	10/49(0.20)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	1.021	0.835
Lower Limit	---	---	0.294	0.353
Upper Limit	---	---	3.548	1.955
Weeks to First Observed Tumor	96	78	94	96
Hematopoietic System: Leukemia or Malignant Lymphoma[b]	4/48(0.08)	2/46(0.04)	5/49(0.10)	4/49(0.08)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	1.224	1.878
Lower Limit	---	---	0.281	0.284
Upper Limit	---	---	5.823	19.990
Weeks to First Observed Tumor	96	97	94	72

TABLE 5 (CONCLUDED)

TOPOGRAPHY:MORPHOLOGY	LOW DOSE CONTROL	HIGH DOSE CONTROL	LOW DOSE	HIGH DOSE
Liver: Hepatocellular Carcinoma[b]	7/48(0.15)	10/45(0.22)	8/48(0.17)	16/49(0.33)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	1.143	1.469
Lower Limit	---	---	0.394	0.705
Upper Limit	---	---	3.411	3.233
Weeks to First Observed Tumor	78	93	94	77
Liver: Hepatocellular Carcinoma or Neoplastic Nodule[b]	7/48(0.15)	10/45(0.22)	8/48(0.17)	18/49(0.37)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	1.143	1.653
Lower Limit	---	---	0.394	0.817
Upper Limit	---	---	3.411	3.558
Weeks to First Observed Tumor	78	93	94	77

[a]Treated groups received doses of 0.06 or 0.10 percent in feed.

[b]Number of tumor-bearing animals/number of animals examined at site (proportion).

[c]The probability level for the Fisher exact test for the comparison of a treated group with its control group is given beneath the incidence of tumors in the treated group when $P < 0.05$; otherwise, not significant (N.S.) is indicated. A negative designation (N) indicates a lower incidence in the treated group than in the control group.

[d]The 95% confidence interval on the relative risk of the treated group to the control group.

TABLE 6

ANALYSES OF THE INCIDENCE OF PRIMARY TUMORS AT SPECIFIC SITES IN FEMALE MICE TREATED WITH 2,5-TOLUENEDIAMINE SULFATE[a]

TOPOGRAPHY:MORPHOLOGY	LOW DOSE CONTROL	HIGH DOSE CONTROL	LOW DOSE	HIGH DOSE
Lung: Alveolar/Bronchiolar Adenoma or Alveolar/Bronchiolar Carcinoma[b]	4/46(0.09)	1/45(0.02)	6/42(0.14)	8/45(0.17)
P Values[c]	---	---	N.S.	P = 0.016
Relative Risk (Control)[d]	---	---	1.643	7.826
Lower Limit	---	---	0.419	1.118
Upper Limit	---	---	7.390	338.408
Weeks to First Observed Tumor	96	98	94	78
Hematopoietic System: Leukemia or Malignant Lymphoma[b]	5/48(0.10)	12/46(0.26)	4/45(0.09)	8/47(0.17)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	0.853	0.653
Lower Limit	---	---	0.180	0.256
Upper Limit	---	---	3.711	1.568
Weeks to First Observed Tumor	96	95	94	78
Liver: Hepatocellular Carcinoma[b]	1/47(0.02)	4/45(0.09)	2/42(0.05)	4/46(0.09)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	2.238	1.000
Lower Limit	---	---	0.121	0.198
Upper Limit	---	---	128.900	5.050
Weeks to First Observed Tumor	96	78	93	94

TABLE 6 (CONCLUDED)

TOPOGRAPHY:MORPHOLOGY	LOW DOSE CONTROL	HIGH DOSE CONTROL	LOW DOSE	HIGH DOSE
Pituitary: Adenoma NOS or Carcinoma NOS[b]	3/42(0.07)	6/37(0.16)	1/38(0.03)	0/38(0.00)
P Values[c]	---	---	N.S.	P = 0.012(N)
Relative Risk (Control)[d]	---	---	0.368	0.000
Lower Limit	---	---	0.007	0.000
Upper Limit	---	---	4.349	0.602
Weeks to First Observed Tumor	96	98	94	---
Ovary: Papillary Cystadenoma NOS or Tubular Adenoma[b]	1/45(0.02)	0/41(0.00)	0/36(0.00)	2/39(0.05)
P Values[c]	---	---	N.S.	N.S.
Relative Risk (Control)[d]	---	---	0.000	Infinite
Lower Limit	---	---	0.000	0.313
Upper Limit	---	---	23.152	Infinite
Weeks to First Observed Tumor	78	---	---	97

[a]Treated groups received doses of 0.06 or 0.10 percent in feed.

[b]Number of tumor-bearing animals/number of animals examined at site (proportion).

[c]The probability level for the Fisher exact test for the comparison of a treated group with its control group is given beneath the incidence of tumors in the treated group when $P < 0.05$; otherwise, not significant (N.S.) is indicated. A negative designation (N) indicates a lower incidence in the treated group than in the control group.

[d]The 95% confidence interval on the relative risk of the treated group to the control group.

the combined incidence in females, the Fisher exact test comparing high dose to high dose control was significant (P = 0.016). In historical data collected by this laboratory for the NCI Carcinogenesis Testing Program 12/350 (3 percent) of the untreated B6C3F1 female mice had an alveolar/bronchiolar adenoma, alveolar/bronchiolar carcinoma, alveolar-cell adenocarcinoma, or an adenoma NOS of the lung/alveoli--compared to the 4/46 (9 percent), 1/45 (2 percent), 6/42 (14 percent), and 8/45 (17 percent) observed in the low dose control, high dose control, low dose, and high dose groups, respectively, in this bioassay.

Based upon these results the statistical conclusion is that the administration of 2,5-toluenediamine sulfate was associated with the incidence of alveolar/bronchiolar neoplasms in female B6C3F1 mice.

For females the Fisher exact test indicated a significantly (P = 0.012) lower combined incidence of pituitary adenomas NOS or pituitary carcinomas NOS in the high dose group than in the high dose control. The low dose comparison was not significant.

To provide additional insight into the possible carcinogenicity of this compound, 95 percent confidence intervals on the relative risk have been estimated and entered in the tables based upon the observed tumor incidence rates. In many of the intervals shown in Tables 5 and 6, the value one is included; this indicates the absence of statistically significant results. It should also be noted that many of the confidence intervals have an upper limit greater than

one, indicating the theoretical possibility of tumor induction in mice by 2,5-toluenediamine sulfate that could not be established under the conditions of this test.

V. DISCUSSION

There was no significant association between compound administration and accelerated mortality in either sex of either species. Adequate numbers of animals in all groups survived sufficiently long to be at risk from late-developing tumors.

Although the incidence of interstitial-cell neoplasms of the testis was statistically significant in each dosed male rat group, development of these tumors was not considered attributable to compound administration since spontaneous incidence of these neoplasms in male Fischer 344 rats is both high and variable. It should also be noted that control rats were housed in a separate room from dosed rats. There were no other neoplasms occurring in male rats at statistically significant incidences, and none of the incidences of neoplasms observed in female rats were statistically significant.

The only site of significantly increased tumor incidence among dosed female mice was the lungs. The combined incidence of alveolar/bronchiolar adenomas and alveolar/bronchiolar carcinomas was statistically significant for the high dose group. The combined incidences of these tumors in both high and low dose female mouse groups were elevated relative to historical controls. However, it should be noted that high dose control mice were housed in a separate room from dosed mice and received in separate shipments from dosed mice. Because of these factors, this increased incidence does not provide

sufficient evidence of a compound-related effect. No significant increase in tumor incidence was observed among dosed male mice.

Under the conditions of this bioassay, sufficient evidence was not provided to conclusively demonstrate the carcinogenicity of 2,5-toluenediamine sulfate in either Fischer 344 rats or B6C3F1 mice.

VI. BIBLIOGRAPHY

Ames, B.N., H.O. Kammen, and E. Yamasaki, "Hair Dyes are Mutagenic: Identification of a Variety of Mutagenic Ingredients." Proceedings of the National Academy of Science U.S.A. 72:2423-2427, 1975.

Anthony, H.M., and G.M. Thomas, "Tumors of the Urinary Bladder: An Analysis of the Occupations of 1,030 Patients in Leeds, England." Journal of the National Cancer Institute 45:879-895, 1970.

Armitage, P., Statistical Methods in Medical Research, Chapter 14. J. Wiley & Sons, New York, 1971.

Berenblum, I., editor, Carcinogenicity Testing. International Union Against Cancer, Technical Report Series, Vol. 2. International Union Against Cancer, Geneva, 1969.

Chemical Abstracts Service, The Chemical Abstracts Service (CAS) Ninth Collective Index, Volumes 76-85, 1972-1976. American Chemical Society, Washington, D.C., 1977.

Cockrell, B.Y. and F.M. Garner, "Interstitial-Cell Tumors of the Testis in Rats." Comparative Pathology Bulletin 8:2-4, 1976.

Cox, D.R., Analysis of Binary Data, Chapters 4 and 5. Methuen and Co., Ltd., London, 1970.

Cox, D.R., "Regression Models and Life-Tables." Journal of the Royal Statistical Society, Series "B" 34:187-220, 1972.

Gart, J.J., "The Comparison of Proportions: A Review of Significance Tests, Confidence Limits, and Adjustments for Stratification." International Statistical Institute Review 39:148-169, 1971.

Hawley, G.G., editor, The Condensed Chemical Dictionary, 8th edition. Van Nostrand Reinhold Company, New York, 1971.

Hoover, R. and J. Fraumeni, Jr., "Cancer Mortality in U.S. Counties with Chemical Industries." Environmental Research 9:196-207, 1975.

Kaplan, E.L., and P. Meier, "Nonparametric Estimation from Incomplete Observations." Journal of the American Statistical Association 53:457-481, 1958.

Kiese, M., and E. Rauscher, "The Absorption of p-toluenediamine Through Human Skin in Hair Dyeing." Toxicology and Applied Pharmacology 13:325-331, 1968.

Kiese, M., M. Rachor, and E. Rauscher, "The Absorption of Some Phenylenediamines Through the Skin of Dogs." Toxicology and Applied Pharmacology 12:495-507, 1968.

Linhart, M.S., J.A. Cooper, R.L. Martin, N.P. Page, and J. A. Peters, "Carcinogenesis Bioassay Data System." Computers and Biomedical Research 7:230-248, 1974.

Miller, R.G., Simultaneous Statistical Inference. McGraw-Hill Book Co., New York, 1966.

Saffiotti, U., R. Montesano, A.R. Sellakumar, F. Cefis, and D.G. Kaufman, "Respiratory Tract Carcinogenesis in Hamsters Induced by Different Numbers of Administration of Benzo (a) Pyrene and Ferric Oxide." Cancer Research 32:1073-1079, 1972.

Sax, N.I. Dangerous Properties of Industrial Materials. Van Nostrand Reinhold Company, New York, 1975.

Society of Dyers and Colourists, Colour Index, 3rd edition, Volume 3. Yorkshire, England, p. 3260, 1971.

Staats, E.B., Comptroller General of the United States. Cancer and Coal Tar Hair Dyes: An Unregulated Hazard to Consumers. Report to the Subcommittee on Oversight and Investigations, Committee on Interstate and Foreign Commerce, House of Representatives, Washington, D.C., December 6, 1977.

Tarone, R. E., "Tests for Trend in Life-Table Analysis." Biometrika 62:679-682, 1975.

U.S. International Trade Commission, Imports of Benzenoid Chemicals and Products, 1974. USITC Publication 762, U.S. Government Printing Office, Washington, D.C., 1976.

Wynder, E.L., J. Onderdonk, and N. Mantel, "An Epidemiological Investigation of Cancer of the Bladder." Cancer 161:1388-1407, 1963.

APPENDIX A

SUMMARY OF THE INCIDENCE OF NEOPLASMS IN RATS TREATED WITH 2,5-TOLUENEDIAMINE SULFATE

TABLE A1
SUMMARY OF THE INCIDENCE OF NEOPLASMS IN MALE RATS
TREATED WITH 2,5-TOLUENEDIAMINE SULFATE

	HIGH DOSE CONTROL (UNTR) 01-0084	LOW DOSE CONTROL (UNTR) 01-0037	LOW DOSE 01-0039	HIGH DOSE 01-0090
ANIMALS INITIALLY IN STUDY	25	50	50	50
ANIMALS NECROPSIED	25	46	49	49
ANIMALS EXAMINED HISTOPATHOLOGICALLY**	25	46	48	49
INTEGUMENTARY SYSTEM				
*SKIN	(25)	(46)	(49)	(49)
SQUAMOUS CELL CARCINOMA			1 (2%)	
*SUBCUT TISSUE	(25)	(46)	(49)	(49)
FIBROMA			1 (2%)	1 (2%)
LIPOMA			1 (2%)	
HEMANGIOSARCOMA				1 (2%)
NEUROFIBROMA				1 (2%)
RESPIRATORY SYSTEM				
#TRACHEA	(11)	(45)	(46)	(47)
ADENOCARCINOMA, NOS, METASTATIC		1 (2%)		
#LUNG	(25)	(46)	(48)	(49)
UNDIFFERENTIATED CARCINOMA METAS				1 (2%)
ADENOCARCINOMA, NOS, METASTATIC		1 (2%)		
ALVEOLAR/BRONCHIOLAR ADENOMA	2 (8%)		1 (2%)	
ALVEOLAR/BRONCHIOLAR CARCINOMA	1 (4%)			
PAPILLARY ADENOCARCINOMA, METAST			1 (2%)	
PHEOCHROMOCYTOMA, METASTATIC	1 (4%)			
HEMATOPOIETIC SYSTEM				
*MULTIPLE ORGANS	(25)	(46)	(49)	(49)
MALIGNANT LYMPHOMA, NOS			1 (2%)	
MALIG.LYMPHOMA, HISTIOCYTIC TYPE			1 (2%)	
UNDIFFERENTIATED LEUKEMIA	2 (8%)	1 (2%)		
MYELOMONOCYTIC LEUKEMIA			2 (4%)	5 (10%)
LYMPHOCYTIC LEUKEMIA	2 (8%)		1 (2%)	1 (2%)
MONOCYTIC LEUKEMIA		1 (2%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED
** EXCLUDES PARTIALLY AUTOLYZED ANIMALS

TABLE A1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 01-0084	LOW DOSE CONTROL (UNTR) 01-0037	LOW DOSE 01-0039	HIGH DOSE 01-0090
#LYMPH NODE	(24)	(38)	(39)	(43)
ADENOCARCINOMA, NOS, METASTATIC		1 (3%)		
#MEDIASTINAL L.NODE	(24)	(38)	(39)	(43)
UNDIFFERENTIATED CARCINOMA METAS				1 (2%)
#ABDOMINAL LYMPH NODE	(24)	(38)	(39)	(43)
UNDIFFERENTIATED CARCINOMA METAS				1 (2%)
#LIVER	(25)	(46)	(48)	(49)
MYELOMONOCYTIC LEUKEMIA				1 (2%)
GRANULOCYTIC LEUKEMIA				1 (2%)
#THYMUS	(22)	(38)	(39)	(28)
THYMOMA			1 (3%)	
CIRCULATORY SYSTEM				
NONE				
DIGESTIVE SYSTEM				
#LIVER	(25)	(46)	(48)	(49)
UNDIFFERENTIATED CARCINOMA METAS				1 (2%)
NEOPLASTIC NODULE			1 (2%)	
HEPATOCELLULAR CARCINOMA			1 (2%)	
#PANCREAS	(25)	(42)	(48)	(48)
UNDIFFERENTIATED CARCINOMA				1 (2%)
ADENOMA, NOS				1 (2%)
*STOMACH	(24)	(45)	(47)	(49)
SQUAMOUS CELL PAPILLOMA	1 (4%)			
BASAL-CELL CARCINOMA	1 (4%)			
*JEJUNUM	(24)	(43)	(48)	(48)
CYSTADENOCARCINOMA, NOS				1 (2%)
*COLON	(24)	(43)	(47)	(48)
ADENOCARCINOMA, NOS				1 (2%)
URINARY SYSTEM				
#KIDNEY	(24)	(46)	(48)	(49)
TUBULAR-CELL ADENOMA				1 (2%)

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE A1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 01-0084	LOW DOSE CONTROL (UNTR) 01-0037	LOW DOSE 01-0039	HIGH DOSE 01-0090
#URINARY BLADDER	(23)	(42)	(48)	(45)
PAPILLOMA, NOS			1 (2%)	1 (2%)
TRANSITIONAL-CELL CARCINOMA			1 (2%)	
ENDOCRINE SYSTEM				
#PITUITARY	(21)	(41)	(45)	(40)
ADENOMA, NOS	1 (5%)	2 (5%)	3 (7%)	3 (8%)
CHROMOPHOBE ADENOMA		10 (24%)		
BASOPHIL ADENOMA	2 (10%)			
#ADRENAL	(25)	(43)	(48)	(48)
ADENOCARCINOMA, NOS, METASTATIC		1 (2%)		
PHECCHROMOCYTOMA	2 (8%)	6 (14%)	2 (4%)	1 (2%)
PHECCHROMOCYTOMA, MALIGNANT	2 (8%)			
#THYROID	(23)	(45)	(46)	(47)
ADENOMA, NOS		1 (2%)		
ADENOCARCINOMA, NOS		2 (4%)		1 (2%)
PAPILLARY ADENOCARCINOMA			3 (7%)	
C-CELL ADENOMA		1 (2%)		
C-CELL CARCINOMA			1 (2%)	1 (2%)
CYSTADENOCARCINOMA, NOS				1 (2%)
PAPILLARY CYSTADENOCARCINOMA,NOS				1 (2%)
#PANCREATIC ISLETS	(25)	(42)	(48)	(48)
ISLET-CELL ADENOMA	2 (8%)	2 (5%)	4 (8%)	1 (2%)
REPRODUCTIVE SYSTEM				
*MAMMARY GLAND	(25)	(46)	(49)	(49)
FIBROADENOMA	1 (4%)		1 (2%)	
*PREPUTIAL GLAND	(25)	(46)	(49)	(49)
CARCINOMA,NOS	1 (4%)			2 (4%)
ADENCMA, NOS	1 (4%)			
#PROSTATE	(23)	(45)	(47)	(46)
PARAGANGLIOMA, NOS		1 (2%)		
#TESTIS	(24)	(45)	(48)	(48)
INTERSTITIAL-CELL TUMOR	19 (79%)	33 (73%)	43 (90%)	47 (98%)

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE A1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 01-0084	LOW DOSE CONTROL (UNTR) 01-0037	LOW DOSE 01-0039	HIGH DOSE 01-0090
*SCROTUM	(25)	(46)	(49)	(49)
LEIOMYOMA				1 (2%)
NERVOUS SYSTEM				
#BRAIN	(25)	(44)	(48)	(48)
ASTROCYTOMA		1 (2%)		
SPECIAL SENSE ORGANS				
*EAR	(25)	(46)	(49)	(49)
FIBROMA				1 (2%)
*EAR CANAL	(25)	(46)	(49)	(49)
SQUAMOUS CELL CARCINOMA	1 (4%)			
MUSCULOSKELETAL SYSTEM				
NONE				
BODY CAVITIES				
*BODY CAVITIES	(25)	(46)	(49)	(49)
MESOTHELIOMA, NOS			1 (2%)	
*MEDIASTINUM	(25)	(46)	(49)	(49)
ALVEOLAR/BRONCHIOLAR CA, METASTA	1 (4%)			
*PLEURA	(25)	(46)	(49)	(49)
ALVEOLAR/BRONCHIOLAR CA, METASTA	1 (4%)			
ALL OTHER SYSTEMS				
NONE				

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE A1 (CONCLUDED)

	HIGH DOSE CONTROL (UNTR) 01-0084	LOW DOSE CONTROL (UNTR) 01-0037	LOW DOSE 01-0039	HIGH DOSE 01-0090
ANIMAL DISPOSITION SUMMARY				
ANIMALS INITIALLY IN STUDY	25	50	50	50
NATURAL DEATH@	3	6	5	3
MORIBUND SACRIFICE	4	2	5	11
SCHEDULED SACRIFICE	5	15	5	
ACCIDENTALLY KILLED				
TERMINAL SACRIFICE	13	27	35	36
ANIMAL MISSING				
@ INCLUDES AUTOLYZED ANIMALS				
TUMOR SUMMARY				
TOTAL ANIMALS WITH PRIMARY TUMORS*	22	34	45	48
TOTAL PRIMARY TUMORS	41	61	72	77
TOTAL ANIMALS WITH BENIGN TUMORS	20	33	44	47
TOTAL BENIGN TUMORS	31	55	58	59
TOTAL ANIMALS WITH MALIGNANT TUMORS	9	5	9	17
TOTAL MALIGNANT TUMORS	10	5	12	18
TOTAL ANIMALS WITH SECONDARY TUMORS#	2	1	1	1
TOTAL SECONDARY TUMORS	3	4	1	4
TOTAL ANIMALS WITH TUMORS UNCERTAIN-BENIGN OR MALIGNANT		1	2	
TOTAL UNCERTAIN TUMORS		1	2	
TOTAL ANIMALS WITH TUMORS UNCERTAIN-PRIMARY OR METASTATIC				
TOTAL UNCERTAIN TUMORS				

* PRIMARY TUMORS: ALL TUMORS EXCEPT SECONDARY TUMORS
\# SECONDARY TUMORS: METASTATIC TUMORS OR TUMORS INVASIVE INTO AN ADJACENT ORGAN

TABLE A2
SUMMARY OF THE INCIDENCE OF NEOPLASMS IN FEMALE RATS
TREATED WITH 2,5-TOLUENEDIAMINE SULFATE

	HIGH DOSE CONTROL (UNTR) 02-0084	LOW DOSE CONTROL (UNTR) 02-0037	LOW DOSE 02-0039	HIGH DOSE 02-0090
ANIMALS INITIALLY IN STUDY	25	50	50	50
ANIMALS NECROPSIED	23	49	50	50
ANIMALS EXAMINED HISTOPATHOLOGICALLY**	23	49	50	50
INTEGUMENTARY SYSTEM				
*SKIN	(23)	(49)	(50)	(50)
SQUAMOUS CELL CARCINOMA			1 (2%)	
SEBACEOUS ADENOCARCINOMA	1 (4%)			
FIBROSARCOMA				1 (2%)
*SUBCUT TISSUE	(23)	(49)	(50)	(50)
SQUAMOUS CELL CARCINOMA				1 (2%)
RESPIRATORY SYSTEM				
#LUNG	(23)	(49)	(50)	(50)
ADENOCARCINOMA, NOS, METASTATIC		1 (2%)	1 (2%)	
HEPATOCELLULAR CARCINOMA, METAST		1 (2%)		
ALVEOLAR/BRONCHIOLAR ADENOMA	1 (4%)	1 (2%)		1 (2%)
HEMATOPOIETIC SYSTEM				
*MULTIPLE ORGANS	(23)	(49)	(50)	(50)
MALIG.LYMPHOMA, LYMPHOCYTIC TYPE		2 (4%)		
LEUKEMIA,NOS				1 (2%)
UNDIFFERENTIATED LEUKEMIA	2 (9%)			
MYELOMONOCYTIC LEUKEMIA			2 (4%)	8 (16%)
MONOCYTIC LEUKEMIA		2 (4%)		
#PENAL LYMPH NODE	(21)	(41)	(47)	(49)
ADENOCARCINOMA, NOS, METASTATIC		1 (2%)		
#THYMUS	(20)	(42)	(43)	(44)
MALIGNANT LYMPHOMA, NOS				1 (2%)
CIRCULATORY SYSTEM				
NONE				

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED
**EXCLUDES PARTIALLY AUTOLYZED ANIMALS

TABLE A2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 02-0084	LOW DOSE CONTROL (UNTR) 02-0037	LOW DOSE 02-0039	HIGH DOSE 02-0090
DIGESTIVE SYSTEM				
#LIVER	(23)	(49)	(50)	(49)
ADENOCARCINOMA, NOS, METASTATIC		1 (2%)		
NEOPLASTIC NODULE	2 (9%)			
HEPATOCELLULAR CARCINOMA		2 (4%)	1 (2%)	1 (2%)
#STOMACH	(23)	(48)	(50)	(49)
SQUAMOUS CELL PAPILLOMA				1 (2%)
#ILEUM	(23)	(47)	(50)	(49)
LEIOMYOSARCOMA				1 (2%)
URINARY SYSTEM				
#URINARY BLADDER	(22)	(41)	(47)	(50)
PAPILLOMA, NOS				1 (2%)
ENDOCRINE SYSTEM				
#PITUITARY	(21)	(43)	(48)	(43)
ADENOMA, NOS	1 (5%)	3 (7%)	17 (35%)	10 (23%)
ADENOCARCINOMA, NOS		2 (5%)		
CHROMOPHOBE ADENOMA	7 (33%)	15 (35%)		
#ADRENAL	(23)	(46)	(50)	(48)
PHEOCHROMOCYTOMA	2 (9%)	2 (4%)	1 (2%)	1 (2%)
PHEOCHROMOCYTOMA, MALIGNANT	1 (4%)			
#THYROID	(21)	(47)	(48)	(49)
ADENOMA, NOS		1 (2%)		
ADENOCARCINOMA, NOS		2 (4%)		1 (2%)
C-CELL ADENOMA	2 (10%)	1 (2%)		
C-CELL CARCINOMA	1 (5%)		3 (6%)	
#THYROID FOLLICLE	(21)	(47)	(48)	(49)
PAPILLARY CYSTADENOCARCINOMA,NOS	1 (5%)			
REPRODUCTIVE SYSTEM				
*MAMMARY GLAND	(23)	(49)	(50)	(50)
ADENOMA, NOS		1 (2%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE A2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 02-0084	LOW DOSE CONTROL (UNTR) 02-0037	LOW DOSE 02-0039	HIGH DOSE 02-0090
ADENOCARCINOMA, NOS	2 (9%)	1 (2%)		1 (2%)
PAPILLARY ADENOCARCINOMA			1 (2%)	
PAPILLARY CYSTADENOMA, NOS				1 (2%)
PAPILLARY CYSTADENOCARCINOMA,NOS		1 (2%)		
INFILTRATING DUCT CARCINOMA	1 (4%)			
FIBROADENOMA	4 (17%)	4 (8%)	6 (12%)	9 (18%)
*CLITORAL GLAND	(23)	(49)	(50)	(50)
CARCINOMA,NOS				1 (2%)
#UTERUS	(23)	(48)	(49)	(45)
ADENOCARCINOMA, NOS		4 (8%)	1 (2%)	1 (2%)
ENDOMETRIAL STROMAL POLYP	6 (26%)	10 (21%)	7 (14%)	7 (16%)
HEMANGIOMA			1 (2%)	
#UTERUS/ENDOMETRIUM	(23)	(48)	(49)	(45)
ADENOCARCINOMA, NOS			2 (4%)	
#OVARY	(22)	(47)	(50)	(46)
TUBULAR ADENOMA				1 (2%)
NERVOUS SYSTEM				
NONE				
SPECIAL SENSE ORGANS				
*EAR	(23)	(49)	(50)	(50)
SQUAMOUS CELL CARCINOMA			2 (4%)	
*EAR CANAL	(23)	(49)	(50)	(50)
FIBROMA		1 (2%)		
MUSCULOSKELETAL SYSTEM				
NONE				
BODY CAVITIES				
*BODY CAVITIES	(23)	(49)	(50)	(50)
MESOTHELIOMA, MALIGNANT		1 (2%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE A2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 02-0084	LOW DOSE CONTROL (UNTR) 02-0037	LOW DOSE 02-0039	HIGH DOSE 02-0090
*ABDOMINAL CAVITY	(23)	(49)	(50)	(50)
SARCOMA, NOS			1 (2%)	1 (2%)
LEIOMYOSARCOMA			1 (2%)	
*PERITONEUM	(23)	(49)	(50)	(50)
ADENOCARCINOMA, NOS, METASTATIC			1 (2%)	
LIPOMA				1 (2%)
ALL OTHER SYSTEMS				
DIAPHRAGM				
RHABDOMYOSARCOMA				1
ANIMAL DISPOSITION SUMMARY				
ANIMALS INITIALLY IN STUDY	25	50	50	50
NATURAL DEATH@	3	5	2	
MORIBUND SACRIFICE	5	7	11	11
SCHEDULED SACRIFICE	5	15	5	
ACCIDENTALLY KILLED				
TERMINAL SACRIFICE	12	23	32	39
ANIMAL MISSING				

@ INCLUDES AUTOLYZED ANIMALS

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE A2 (CONCLUDED)

	HIGH DOSE CONTROL (UNTR) 02-0084	LOW DOSE CONTROL (UNTR) 02-0037	LOW DOSE 02-0039	HIGH DOSE 02-0090
TUMOR SUMMARY				
TOTAL ANIMALS WITH PRIMARY TUMORS*	19	32	34	33
TOTAL PRIMARY TUMORS	34	56	47	53
TOTAL ANIMALS WITH BENIGN TUMORS	18	27	28	23
TOTAL BENIGN TUMORS	23	39	32	33
TOTAL ANIMALS WITH MALIGNANT TUMORS	8	15	14	17
TOTAL MALIGNANT TUMORS	9	17	15	20
TOTAL ANIMALS WITH SECONDARY TUMORS#		2	1	
TOTAL SECONDARY TUMORS		4	2	
TOTAL ANIMALS WITH TUMORS UNCERTAIN-BENIGN OR MALIGNANT	2			
TOTAL UNCERTAIN TUMORS	2			
TOTAL ANIMALS WITH TUMORS UNCERTAIN-PRIMARY OR METASTATIC				
TOTAL UNCERTAIN TUMORS				

* PRIMARY TUMORS: ALL TUMORS EXCEPT SECONDARY TUMORS
\# SECONDARY TUMORS: METASTATIC TUMORS OR TUMORS INVASIVE INTO AN ADJACENT ORGAN

APPENDIX B

SUMMARY OF THE INCIDENCE OF NEOPLASMS IN MICE TREATED WITH 2,5-TOLUENEDIAMINE SULFATE

TABLE B1
SUMMARY OF THE INCIDENCE OF NEOPLASMS IN MALE MICE
TREATED WITH 2,5-TOLUENEDIAMINE SULFATE

	HIGH DOSE CONTROL (UNTR) 05-0077	LOW DOSE CONTROL (UNTR) 05-0037	LOW DOSE 05-0039	HIGH DOSE 05-0089
ANIMALS INITIALLY IN STUDY	50	50	50	50
ANIMALS NECROPSIED	46	48	49	49
ANIMALS EXAMINED HISTOPATHOLOGICALLY**	45	48	48	49
INTEGUMENTARY SYSTEM				
*SUBCUT TISSUE	(46)	(48)	(49)	(49)
OSTEOMA			1 (2%)	
RESPIRATORY SYSTEM				
#LUNG	(45)	(48)	(47)	(49)
HEPATOCELLULAR CARCINOMA, METAST	1 (2%)			2 (4%)
ALVEOLAR/BRONCHIOLAR ADENOMA	7 (16%)		5 (11%)	5 (10%)
ALVEOLAR/BRONCHIOLAR CARCINOMA	4 (9%)	6 (13%)	1 (2%)	5 (10%)
HEMATOPOIETIC SYSTEM				
*MULTIPLE ORGANS	(46)	(48)	(49)	(49)
MALIGNANT LYMPHOMA, NOS		2 (4%)	4 (8%)	2 (4%)
MALIG.LYMPHOMA, HISTIOCYTIC TYPE		2 (4%)		
#SPLEEN	(45)	(47)	(48)	(43)
HEMANGIOMA		1 (2%)		
MALIG.LYMPHOMA, HISTIOCYTIC TYPE	1 (2%)		1 (2%)	
#MANDIBULAR L. NODE	(35)	(44)	(44)	(36)
MALIG.LYMPHOMA, HISTIOCYTIC TYPE	1 (3%)			
#MESENTERIC L. NODE	(35)	(44)	(44)	(36)
MALIGNANT LYMPHOMA, NOS				1 (3%)
#LIVER	(45)	(48)	(48)	(49)
MALIG.LYMPHOMA, HISTIOCYTIC TYPE				1 (2%)
CIRCULATORY SYSTEM				
NONE				

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED
** EXCLUDES PARTIALLY AUTOLYZED ANIMALS

TABLE B1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 05-0077	LOW DOSE CONTROL (UNTR) 05-0037	LOW DOSE 05-0039	HIGH DOSE 05-0089
DIGESTIVE SYSTEM				
*LIVER	(45)	(48)	(48)	(49)
NEOPLASTIC NODULE				2 (4%)
HEPATOCELLULAR CARCINOMA	10 (22%)	7 (15%)	8 (17%)	16 (33%)
HEMANGIOMA		1 (2%)		
*STOMACH	(42)	(47)	(46)	(48)
SQUAMOUS CELL PAPILLOMA	1 (2%)			
SQUAMOUS CELL CARCINOMA		1 (2%)		
URINARY SYSTEM				
NONE				
ENDOCRINE SYSTEM				
#ADRENAL	(43)	(45)	(45)	(44)
PHEOCHROMOCYTOMA				1 (2%)
#THYROID	(40)	(47)	(46)	(46)
FOLLICULAR-CELL ADENOMA		1 (2%)		
REPRODUCTIVE SYSTEM				
NONE				
NERVOUS SYSTEM				
NONE				
SPECIAL SENSE ORGANS				
*HARDERIAN GLAND	(46)	(48)	(49)	(49)
ADENOMA, NOS				1 (2%)
PAPILLARY CYSTADENOMA, NOS			1 (2%)	
*EAR CANAL	(46)	(48)	(49)	(49)
SQUAMOUS CELL CARCINOMA	1 (2%)			
MUSCULOSKELETAL SYSTEM				
NONE				

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE B1 (CONCLUDED)

	HIGH DOSE CONTROL (UNTR) 05-0077	LOW DOSE CONTROL (UNTR) 05-0037	LOW DOSE 05-0039	HIGH DOSE 05-0089
BODY CAVITIES				
NONE				
ALL OTHER SYSTEMS				
NONE				
ANIMAL DISPOSITION SUMMARY				
ANIMALS INITIALLY IN STUDY	50	50	50	50
NATURAL DEATH@	7	3	3	7
MORIBUND SACRIFICE	1			1
SCHEDULED SACRIFICE	5	5		5
ACCIDENTALLY KILLED				
TERMINAL SACRIFICE	37	42	47	37
ANIMAL MISSING				
@ INCLUDES AUTOLYZED ANIMALS				
TUMOR SUMMARY				
TOTAL ANIMALS WITH PRIMARY TUMORS*	21	17	19	27
TOTAL PRIMARY TUMORS	25	21	21	34
TOTAL ANIMALS WITH BENIGN TUMORS	8	2	7	7
TOTAL BENIGN TUMORS	8	3	7	7
TOTAL ANIMALS WITH MALIGNANT TUMORS	15	15	13	23
TOTAL MALIGNANT TUMORS	17	18	14	25
TOTAL ANIMALS WITH SECONDARY TUMORS#	1			2
TOTAL SECONDARY TUMORS	1			2
TOTAL ANIMALS WITH TUMORS UNCERTAIN-BENIGN OR MALIGNANT				2
TOTAL UNCERTAIN TUMORS				2
TOTAL ANIMALS WITH TUMORS UNCERTAIN-PRIMARY OR METASTATIC				
TOTAL UNCERTAIN TUMORS				

* PRIMARY TUMORS: ALL TUMORS EXCEPT SECONDARY TUMORS
\# SECONDARY TUMORS: METASTATIC TUMORS OR TUMORS INVASIVE INTO AN ADJACENT ORGAN

TABLE B2
SUMMARY OF THE INCIDENCE OF NEOPLASMS IN FEMALE MICE
TREATED WITH 2,5-TOLUENEDIAMINE SULFATE

	HIGH DOSE CONTROL (UNTR) 06-0077	LOW DOSE CONTROL (UNTR) 06-0037	LOW DOSE 06-0039	HIGH DOSE 06-0089
ANIMALS INITIALLY IN STUDY	50	50	50	50
ANIMALS MISSING			1	
ANIMALS NECROPSIED	46	48	45	47
ANIMALS EXAMINED HISTOPATHOLOGICALLY**	46	47	43	46
INTEGUMENTARY SYSTEM				
*SKIN	(46)	(48)	(45)	(47)
FIBROSARCOMA	2 (4%)			
*SUBCUT TISSUE	(46)	(48)	(45)	(47)
SARCOMA, NOS			1 (2%)	
LEIOMYOSARCOMA		1 (2%)		
RESPIRATORY SYSTEM				
#LUNG	(45)	(46)	(42)	(46)
HEPATOCELLULAR CARCINOMA, METAST			1 (2%)	
ALVEOLAR/BRONCHIOLAR ADENOMA	1 (2%)	3 (7%)	6 (14%)	7 (15%)
ALVEOLAR/BRONCHIOLAR CARCINOMA		1 (2%)		1 (2%)
HEMATOPOIETIC SYSTEM				
*MULTIPLE ORGANS	(46)	(48)	(45)	(47)
MALIGNANT LYMPHOMA, NOS	3 (7%)	1 (2%)	1 (2%)	3 (6%)
MALIG.LYMPHOMA, UNDIFFER-TYPE	1 (2%)			
MALIG.LYMPHOMA, HISTIOCYTIC TYPE	6 (13%)	2 (4%)	3 (7%)	1 (2%)
LYMPHOCYTIC LEUKEMIA	1 (2%)			
#SPLEEN	(43)	(46)	(42)	(42)
HEMANGIOSARCOMA		1 (2%)	1 (2%)	
MALIG.LYMPHOMA, HISTIOCYTIC TYPE		1 (2%)		2 (5%)
#LYMPH NODE	(41)	(39)	(37)	(32)
MALIGNANT LYMPHOMA, NOS				1 (3%)
#PEYERS PATCH	(43)	(44)	(42)	(42)
MALIG.LYMPHOMA, HISTIOCYTIC TYPE	1 (2%)			

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED
** EXCLUDES PARTIALLY AUTOLYZED ANIMALS

TABLE B2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 06-0077	LOW DOSE CONTROL (UNTR) 06-0037	LOW DOSE 06-0039	HIGH DOSE 06-0089
#DUODENUM	(43)	(44)	(42)	(42)
MALIGNANT LYMPHOMA, NOS				1 (2%)
#THYMUS	(27)	(31)	(29)	(29)
MALIGNANT LYMPHOMA, NOS		1 (3%)		
CIRCULATORY SYSTEM				
*PULMONARY ARTERY	(46)	(48)	(45)	(47)
ADENOCARCINOMA, NOS, METASTATIC			1 (2%)	
DIGESTIVE SYSTEM				
#LIVER	(45)	(47)	(42)	(46)
HEPATOCELLULAR CARCINOMA	4 (9%)	1 (2%)	2 (5%)	4 (9%)
FIBROSARCOMA		1 (2%)		
*BILE DUCT	(46)	(48)	(45)	(47)
BILE DUCT CARCINOMA				1 (2%)
#STOMACH	(42)	(44)	(42)	(43)
SQUAMOUS CELL PAPILLOMA	3 (7%)	1 (2%)		
#JEJUNUM	(43)	(44)	(42)	(42)
ADENOCARCINOMA, NOS			1 (2%)	
#COLON	(41)	(40)	(41)	(43)
LEIOMYOSARCOMA		1 (3%)		
URINARY SYSTEM				
NONE				
ENDOCRINE SYSTEM				
#PITUITARY	(37)	(42)	(38)	(38)
CARCINOMA,NOS		1 (2%)		
ADENOMA, NOS	6 (16%)	2 (5%)	1 (3%)	
#ADRENAL	(43)	(45)	(38)	(41)
CORTICAL ADENOMA	1 (2%)			

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE B2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 06-0077	LOW DOSE CONTROL (UNTR) 06-0037	LOW DOSE 06-0039	HIGH DOSE 06-0089
PHECCHROMOCYTOMA		1 (2%)		
#THYROID	(30)	(43)	(36)	(34)
PAPILLARY ADENOCARCINOMA			1 (3%)	
#PANCREATIC ISLETS	(41)	(44)	(42)	(42)
ISLET-CELL ADENOMA	1 (2%)			
REPRODUCTIVE SYSTEM				
*MAMMARY GLAND	(46)	(48)	(45)	(47)
ADENOCARCINOMA, NOS	1 (2%)			
#UTERUS	(43)	(45)	(41)	(42)
LEIOMYOSARCOMA		1 (2%)		
ENDOMETRIAL STROMAL POLYP		3 (7%)		
#OVARY	(41)	(45)	(36)	(39)
PAPILLARY CYSTADENOMA, NOS				1 (3%)
LUTEOMA	1 (2%)			
TUBULAR ADENOMA		1 (2%)		1 (3%)
NERVOUS SYSTEM				
NONE				
SPECIAL SENSE ORGANS				
*HARDERIAN GLAND	(46)	(48)	(45)	(47)
PAPILLARY CYSTADENOMA, NOS				1 (2%)
MUSCULOSKELETAL SYSTEM				
NONE				
BODY CAVITIES				
NONE				
ALL OTHER SYSTEMS				
NONE				

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE B2 (CONCLUDED)

	HIGH DOSE CONTROL (UNTR) 06-0077	LOW DOSE CONTROL (UNTR) 06-0037	LOW DOSE 06-0039	HIGH DOSE 06-0089
ANIMAL DISPOSITION SUMMARY				
ANIMALS INITIALLY IN STUDY	50	50	50	50
NATURAL DEATH@	8	6	9	10
MORIBUND SACRIFICE	2	2	1	2
SCHEDULED SACRIFICE	5	5		5
ACCIDENTALLY KILLED				
TERMINAL SACRIFICE	35	37	39	33
ANIMAL MISSING			1	
@ INCLUDES AUTOLYZED ANIMALS				
TUMOR SUMMARY				
TOTAL ANIMALS WITH PRIMARY TUMORS*	22	20	15	22
TOTAL PRIMARY TUMORS	32	24	17	24
TOTAL ANIMALS WITH BENIGN TUMORS	12	11	7	10
TOTAL BENIGN TUMORS	13	11	7	10
TOTAL ANIMALS WITH MALIGNANT TUMORS	18	11	9	12
TOTAL MALIGNANT TUMORS	19	13	10	14
TOTAL ANIMALS WITH SECONDARY TUMORS#			2	
TOTAL SECONDARY TUMORS			2	
TOTAL ANIMALS WITH TUMORS UNCERTAIN-BENIGN OR MALIGNANT				
TOTAL UNCERTAIN TUMORS				
TOTAL ANIMALS WITH TUMORS UNCERTAIN-PRIMARY OR METASTATIC				
TOTAL UNCERTAIN TUMORS				

* PRIMARY TUMORS: ALL TUMORS EXCEPT SECONDARY TUMORS
\# SECONDARY TUMORS: METASTATIC TUMORS OR TUMORS INVASIVE INTO AN ADJACENT ORGAN

APPENDIX C

SUMMARY OF THE INCIDENCE OF NONNEOPLASTIC LESIONS IN RATS TREATED WITH 2,5-TOLUENEDIAMINE SULFATE

TABLE C1
SUMMARY OF THE INCIDENCE OF NONNEOPLASTIC LESIONS
IN MALE RATS TREATED WITH 2,5-TOLUENEDIAMINE SULFATE

	HIGH DOSE CONTROL (UNTR) 01-0084	LOW DOSE CONTROL (UNTR) 01-0037	LOW DOSE 01-0039	HIGH DOSE 01-0090
ANIMALS INITIALLY IN STUDY	25	50	50	50
ANIMALS NECROPSIED	25	46	49	49
ANIMALS EXAMINED HISTOPATHOLOGICALLY**	25	46	48	49
INTEGUMENTARY SYSTEM				
*SKIN	(25)	(46)	(49)	(49)
EPIDERMAL INCLUSION CYST				1 (2%)
ABSCESS, NOS			1 (2%)	
NECROSIS, NOS	1 (4%)			
*SUBCUT TISSUE	(25)	(46)	(49)	(49)
GRANULATION, TISSUE			1 (2%)	
SCAR				2 (4%)
NECROSIS, NOS			1 (2%)	
RESPIRATORY SYSTEM				
*LARYNX	(25)	(46)	(49)	(49)
INFLAMMATION ACUTE AND CHRONIC	1 (4%)			
INFLAMMATION, CHRONIC	7 (28%)			
#TRACHEA	(11)	(45)	(46)	(47)
INFLAMMATION, NOS	1 (9%)	9 (20%)	1 (2%)	
INFLAMMATION, CHRONIC		10 (22%)		
#LUNG/BRONCHUS	(25)	(46)	(48)	(49)
BRONCHIECTASIS	2 (8%)			
INFLAMMATION, FOCAL	1 (4%)			
ABSCESS, NOS			1 (2%)	
INFLAMMATION, CHRONIC		8 (17%)		
#BRONCHIAL MUCOUS GLA	(25)	(46)	(48)	(49)
ABSCESS, NOS		1 (2%)		
NECROSIS, NOS		1 (2%)		
HYPERPLASIA, ADENOMATOUS		1 (2%)		
#LUNG/BRONCHIOLE	(25)	(46)	(48)	(49)
INFLAMMATION, NOS		1 (2%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED
** EXCLUDES PARTIALLY AUTOLYZED ANIMALS

TABLE C1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 01-0084	LOW DOSE CONTROL (UNTR) 01-0037	LOW DOSE 01-0039	HIGH DOSE 01-0090
INFLAMMATION, FOCAL		1 (2%)		
#LUNG	(25)	(46)	(48)	(49)
EMBRYONAL DUCT CYST			1 (2%)	
ATELECTASIS		1 (2%)		
CONGESTION, NOS		1 (2%)		
EDEMA, NOS		1 (2%)		
INFLAMMATION, NOS		1 (2%)		
INFLAMMATION, FOCAL		3 (7%)		
INFLAMMATION, INTERSTITIAL	2 (8%)	1 (2%)		
INFLAMMATION, SUPPURATIVE		1 (2%)		
BRONCHOPNEUMONIA, ACUTE	1 (4%)			
ABSCESS, NOS	1 (4%)			
PNEUMONIA, CHRONIC MURINE	11 (44%)	1 (2%)		
INFLAMMATION, CHRONIC		1 (2%)		
GRANULOMA, NOS	1 (4%)			
PERIVASCULITIS		5 (11%)		
HEMATOPOIETIC SYSTEM				
#BONE MARROW	(25)	(44)	(48)	(46)
HYPERPLASIA, HEMATOPOIETIC	2 (8%)			
#SPLEEN	(25)	(46)	(48)	(47)
THROMBOSIS, NOS		1 (2%)		
FIBROSIS		1 (2%)		
INFARCT, HEALED		1 (2%)		
HEMOSIDEROSIS	1 (4%)			
RETICULOCYTOSIS		1 (2%)		
HYPERPLASIA, HEMATOPOIETIC	1 (4%)			
HYPERPLASIA, ERYTHROID	1 (4%)	12 (26%)		
HYPERPLASIA, RETICULUM CELL		8 (17%)		
HEMATOPOIESIS			2 (4%)	
ERYTHROPOIESIS			1 (2%)	
#LYMPH NODE	(24)	(38)	(39)	(43)
INFLAMMATION, NOS		1 (3%)		
HYPERPLASIA, NOS		1 (3%)		
PLASMACYTOSIS	1 (4%)			
HYPERPLASIA, RETICULUM CELL		3 (8%)		
#MEDIASTINAL L.NODE	(24)	(38)	(39)	(43)
PLASMACYTOSIS		1 (3%)		
#RENAL LYMPH NODE	(24)	(38)	(39)	(43)
HEMOSIDEROSIS				1 (2%)

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE C1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 01-0084	LOW DOSE CONTROL (UNTR) 01-0037	LOW DOSE 01-0039	HIGH DOSE 01-0090
CIRCULATORY SYSTEM				
*LYMPHATIC VESSELS	(25)	(46)	(49)	(49)
INFLAMMATION, NOS		1 (2%)		
#HEART	(25)	(46)	(48)	(48)
PERIARTERITIS	1 (4%)			
#HEART/VENTRICLE	(25)	(46)	(48)	(48)
FIBROSIS			1 (2%)	
#MYOCARDIUM	(25)	(46)	(48)	(48)
INFLAMMATION, NOS		1 (2%)		
INFLAMMATION, INTERSTITIAL		22 (48%)	1 (2%)	
INFLAMMATION, CHRONIC FOCAL		3 (7%)		
FIBROSIS		7 (15%)		1 (2%)
FIBROSIS, FOCAL	1 (4%)			
DEGENERATION, NOS	10 (40%)			
*AORTA	(25)	(46)	(49)	(49)
INFLAMMATION, CHRONIC FOCAL		1 (2%)		
CALCIFICATION, FOCAL	1 (4%)			
*PULMONARY ARTERY	(25)	(46)	(49)	(49)
HYPERTROPHY, NOS		1 (2%)		
DIGESTIVE SYSTEM				
#SALIVARY GLAND	(24)	(38)	(48)	(48)
CYST, NOS			1 (2%)	
#LIVER	(25)	(46)	(48)	(49)
CONGESTION, CHRONIC PASSIVE	1 (4%)			
INFLAMMATION, CHRONIC			1 (2%)	
CHOLANGIOFIBROSIS	1 (4%)			
NECROSIS, FOCAL	1 (4%)	3 (7%)		
NECROSIS, COAGULATIVE		1 (2%)		1 (2%)
METAMORPHOSIS FATTY	4 (16%)	1 (2%)	1 (2%)	1 (2%)
HYPERPLASIA, FOCAL		23 (50%)		
#LIVER/PERIPORTAL	(25)	(46)	(48)	(49)
FIBROSIS		1 (2%)		
#LIVER/HEPATOCYTES	(25)	(46)	(48)	(49)
DEGENERATION, NOS			1 (2%)	1 (2%)

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE C1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 01-0084	LOW DOSE CONTROL (UNTR) 01-0037	LOW DOSE 01-0039	HIGH DOSE 01-0090
HYPERPLASIA, FOCAL			1 (2%)	1 (2%)
*BILE DUCT	(25)	(46)	(49)	(49)
INFLAMMATION, NOS		6 (13%)		
INFLAMMATION, CHRONIC DIFFUSE			1 (2%)	
HYPERPLASIA, NOS	6 (24%)	32 (70%)	6 (12%)	3 (6%)
HYPERPLASIA, FOCAL		1 (2%)		
*PANCREAS	(25)	(42)	(48)	(48)
INFLAMMATION, NOS	1 (4%)	10 (24%)		
HYPERPLASIA, INTRADUCTAL		1 (2%)		
#PANCREATIC DUCT	(25)	(42)	(48)	(48)
HYPERPLASIA, NOS	1 (4%)			
#PANCREATIC ACINUS	(25)	(42)	(48)	(48)
INFLAMMATION, NOS			1 (2%)	
DEGENERATION, NOS				1 (2%)
ATROPHY, NOS		4 (10%)	1 (2%)	
#STOMACH	(24)	(45)	(47)	(49)
EPIDERMAL INCLUSION CYST	1 (4%)	1 (2%)		
ULCER, NOS		2 (4%)		
HYPERPLASIA, NOS		6 (13%)		
HYPERKERATOSIS		1 (2%)		
ACANTHOSIS		1 (2%)		
#GASTRIC MUCOSA	(24)	(45)	(47)	(49)
ULCER, FOCAL				1 (2%)
#PEYERS PATCH	(24)	(43)	(48)	(48)
HYPERPLASIA, NOS	2 (8%)	7 (16%)		
#COLON	(24)	(43)	(47)	(48)
NEMATODIASIS		3 (7%)		
URINARY SYSTEM				
*KIDNEY	(24)	(46)	(48)	(49)
GLOMERULONEPHRITIS, NOS	5 (21%)	33 (72%)		
INFLAMMATION, INTERSTITIAL		1 (2%)	1 (2%)	
NEPHROPATHY	1 (4%)			
NEPHROSIS, NOS	16 (67%)		42 (88%)	46 (94%)
*KIDNEY/CORTEX	(24)	(46)	(48)	(49)
CYST, NOS				1 (2%)

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE C1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 01-0084	LOW DOSE CONTROL (UNTR) 01-0037	LOW DOSE 01-0039	HIGH DOSE 01-0090
HEMORRHAGE				1 (2%)
METAMORPHOSIS FATTY				1 (2%)
#KIDNEY/TUBULE	(24)	(46)	(48)	(49)
NECROSIS, NOS			1 (2%)	
#URINARY BLADDER	(23)	(42)	(48)	(45)
CALCULUS, NOS	3 (13%)			
INFLAMMATION, NOS		1 (2%)		
HYPERPLASIA, EPITHELIAL		3 (7%)	1 (2%)	2 (4%)
ENDOCRINE SYSTEM				
#PITUITARY	(21)	(41)	(45)	(40)
HYPERPLASIA, NOS		3 (7%)		
HYPERPLASIA, CHROMOPHOBE-CELL		2 (5%)		
#PITUITARY/BASOPHIL	(21)	(41)	(45)	(40)
NODULE	1 (5%)			
#ADRENAL	(25)	(43)	(48)	(48)
LIPOIDOSIS				1 (2%)
#ADRENAL CORTEX	(25)	(43)	(48)	(48)
HYPERTROPHY, FOCAL	1 (4%)	1 (2%)		
HYPERPLASIA, NOS		1 (2%)		1 (2%)
#ADRENAL MEDULLA	(25)	(43)	(48)	(48)
NECROSIS, NOS		1 (2%)		
CALCIFICATION, NOS		1 (2%)		
HYPERPLASIA, NODULAR		1 (2%)		
HYPERPLASIA, NOS		6 (14%)		
#THYROID	(23)	(45)	(46)	(47)
HYPERPLASIA, ADENOMATOUS		1 (2%)		
HYPERPLASIA, C-CELL		1 (2%)		1 (2%)
#PANCREATIC ISLETS	(25)	(42)	(48)	(48)
HYPERPLASIA, NOS		2 (5%)		
REPRODUCTIVE SYSTEM				
*MAMMARY GLAND	(25)	(46)	(49)	(49)
HYPERPLASIA, NOS	3 (12%)	5 (11%)	1 (2%)	

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE C1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 01-0084	LOW DOSE CONTROL (UNTR) 01-0037	LOW DOSE 01-0039	HIGH DOSE 01-0090
LACTATION	7 (28%)			
*PREPUTIAL GLAND	(25)	(46)	(49)	(49)
DILATATION, NOS			1 (2%)	
ABSCESS, NOS		1 (2%)		
HYPERPLASIA, NOS		1 (2%)	1 (2%)	
#PROSTATE	(23)	(45)	(47)	(46)
INFLAMMATION, NOS	1 (4%)	21 (47%)		
INFLAMMATION, FOCAL		3 (7%)		
INFLAMMATION, SUPPURATIVE			1 (2%)	
INFLAMMATION, ACUTE			1 (2%)	
ATROPHY, NOS	4 (17%)			
HYPERPLASIA, FOCAL		5 (11%)		
HYPERPLASIA, PAPILLARY		2 (4%)		
METAPLASIA, SQUAMOUS		5 (11%)		
*SEMINAL VESICLE	(25)	(46)	(49)	(49)
ATROPHY, NOS	1 (4%)			2 (4%)
#TESTIS	(24)	(45)	(48)	(48)
CALCIFICATION, FOCAL	4 (17%)			
ATROPHY, NOS	12 (50%)	2 (4%)		1 (2%)
ASPERMATOGENESIS		1 (2%)		
HYPERPLASIA, INTERSTITIAL CELL	2 (8%)	19 (42%)		
#TESTIS/TUBULE	(24)	(45)	(48)	(48)
DEGENERATION, NOS		6 (13%)		1 (2%)
NERVOUS SYSTEM				
#BRAIN	(25)	(44)	(48)	(48)
HEMORRHAGE	2 (8%)			
CALCIFICATION, FOCAL	1 (4%)			
SPECIAL SENSE ORGANS				
*EYE	(25)	(46)	(49)	(49)
INFLAMMATION, NOS			1 (2%)	
*EYE/CORNEA	(25)	(46)	(49)	(49)
ULCER, NOS			1 (2%)	

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE C1 (CONCLUDED)

	HIGH DOSE CONTROL (UNTR) 01-0084	LOW DOSE CONTROL (UNTR) 01-0037	LOW DOSE 01-0039	HIGH DOSE 01-0090
MUSCULOSKELETAL SYSTEM				
*BONE	(25)	(46)	(49)	(49)
OSTEOSCLEROSIS				1 (2%)
*SKELETAL MUSCLE	(25)	(46)	(49)	(49)
CALCIFICATION, FOCAL	1 (4%)			
*CARTILAGE,NOS	(25)	(46)	(49)	(49)
CYST, NOS		1 (2%)		
BODY CAVITIES				
NONE				
ALL OTHER SYSTEMS				
NONE				
SPECIAL MORPHOLOGY SUMMARY				
NO LESION REPORTED			1	
AUTO/NECROPSY/HISTO PERF		1		
AUTO/NECROPSY/NO HISTO			1	
AUTOLYSIS/NO NECROPSY		4	1	1

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE C2
SUMMARY OF THE INCIDENCE OF NONNEOPLASTIC LESIONS
IN FEMALE RATS TREATED WITH 2,5-TOLUENEDIAMINE SULFATE

	HIGH DOSE CONTROL (UNTR) 02-0084	LOW DOSE CONTROL (UNTR) 02-0037	LOW DOSE 02-0039	HIGH DOSE 02-0090
ANIMALS INITIALLY IN STUDY	25	50	50	50
ANIMALS NECROPSIED	23	49	50	50
ANIMALS EXAMINED HISTOPATHOLOGICALLY**	23	49	50	50
INTEGUMENTARY SYSTEM				
NONE				
RESPIRATORY SYSTEM				
*NASAL TURBINATE	(23)	(49)	(50)	(50)
INFLAMMATION, SUPPURATIVE				1 (2%)
*LARYNX	(23)	(49)	(50)	(50)
INFLAMMATION ACUTE AND CHRONIC	1 (4%)			
INFLAMMATION, CHRONIC	3 (13%)			
#TRACHEA	(5)	(48)	(49)	(49)
HEMORRHAGE			1 (2%)	
INFLAMMATION, NOS		9 (19%)	1 (2%)	
INFLAMMATION, CHRONIC		10 (21%)		
POLYP, INFLAMMATORY		1 (2%)		
#LUNG/BRONCHUS	(23)	(49)	(50)	(50)
BRONCHIECTASIS		1 (2%)	1 (2%)	
INFLAMMATION, NOS		1 (2%)		
INFLAMMATION, CHRONIC		9 (18%)		
#LUNG/BRONCHIOLE	(23)	(49)	(50)	(50)
INFLAMMATION, NOS		1 (2%)		
#LUNG	(23)	(49)	(50)	(50)
CYST, NOS			1 (2%)	
INFLAMMATION, NOS		1 (2%)		
INFLAMMATION, FOCAL		7 (14%)		
INFLAMMATION, INTERSTITIAL	3 (13%)	2 (4%)		
PNEUMONIA, CHRONIC MURINE	8 (35%)			
GRANULOMA, FOREIGN BODY	1 (4%)			

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED
**EXCLUDES PARTIALLY AUTOLYZED ANIMALS

TABLE C2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 02-0084	LOW DOSE CONTROL (UNTR) 02-0037	LOW DOSE 02-0039	HIGH DOSE 02-0090
PERIVASCULITIS		6 (12%)		
CALCIFICATION, FOCAL	1 (4%)			
HYPERPLASIA, EPITHELIAL	1 (4%)			
HEMATOPOIETIC SYSTEM				
#BONE MARROW	(22)	(48)	(49)	(45)
HYPERPLASIA, HEMATOPOIETIC	1 (5%)			2 (4%)
#SPLEEN	(23)	(49)	(50)	(49)
HEMATOMA, NOS	1 (4%)			
HEMOSIDEROSIS	2 (9%)			
HYPERPLASIA, NOS		1 (2%)		
HYPERPLASIA, HEMATOPOIETIC	3 (13%)	3 (6%)		
HYPERPLASIA, ERYTHROID	4 (17%)	17 (35%)		
HYPERPLASIA, PLASMA CELL		1 (2%)		
HYPERPLASIA, RETICULUM CELL		11 (22%)		
HEMATOPOIESIS	3 (13%)		1 (2%)	
ERYTHROPOIESIS			3 (6%)	1 (2%)
#LYMPH NODE	(21)	(41)	(47)	(49)
INFLAMMATION, NOS		3 (7%)		
HYPERPLASIA, NOS		2 (5%)		
PLASMACYTOSIS		3 (7%)		
HYPERPLASIA, PLASMA CELL		1 (2%)		
#MEDIASTINAL L.NODE	(21)	(41)	(47)	(49)
HYPERPLASIA, PLASMA CELL				1 (2%)
CIRCULATORY SYSTEM				
#MYOCARDIUM	(23)	(49)	(50)	(50)
INFLAMMATION, NOS		1 (2%)	1 (2%)	
INFLAMMATION, INTERSTITIAL	1 (4%)	24 (49%)	1 (2%)	
INFLAMMATION, CHRONIC			1 (2%)	
FIBROSIS		5 (10%)		
DEGENERATION, NOS	4 (17%)			
*PORTAL VEIN	(23)	(49)	(50)	(50)
THROMBUS, MURAL		1 (2%)		
DIGESTIVE SYSTEM				
#LIVER	(23)	(49)	(50)	(49)
CONGESTION, CHRONIC PASSIVE	1 (4%)			

* NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE C2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 02-0084	LOW DOSE CONTROL (UNTR) 02-0037	LOW DOSE 02-0039	HIGH DOSE 02-0090
INFLAMMATION, CHRONIC			1 (2%)	
INFLAMMATION, FOCAL GRANULOMATOU			1 (2%)	1 (2%)
FIBROSIS		1 (2%)		
CHOLANGIOFIBROSIS	1 (4%)			
PERIVASCULITIS		1 (2%)		
NECROSIS, NOS			1 (2%)	
NECROSIS, FOCAL		4 (8%)	1 (2%)	1 (2%)
NECROSIS, COAGULATIVE		2 (4%)		
METAMORPHOSIS FATTY	2 (9%)	1 (2%)	2 (4%)	1 (2%)
BASOPHILIC CYTO CHANGE	4 (17%)			
HYPERPLASIA, NODULAR		1 (2%)	1 (2%)	
HYPERPLASIA, FOCAL	3 (13%)	22 (45%)	1 (2%)	
ANGIECTASIS		1 (2%)	1 (2%)	
HEMATOPOIESIS			1 (2%)	
#LIVER/CENTRILOBULAR	(23)	(49)	(50)	(49)
NECROSIS, DIFFUSE			1 (2%)	
#LIVER/PERIPORTAL	(23)	(49)	(50)	(49)
METAMORPHOSIS FATTY				1 (2%)
*BILE DUCT	(23)	(49)	(50)	(50)
INFLAMMATION, NOS		5 (10%)		
INFLAMMATION, CHRONIC				1 (2%)
HYPERPLASIA, NOS	2 (9%)	27 (55%)	2 (4%)	1 (2%)
#PANCREAS	(22)	(46)	(50)	(49)
INFLAMMATION, NOS		7 (15%)		
INFLAMMATION, INTERSTITIAL				1 (2%)
PERIARTERITIS			1 (2%)	
#PANCREATIC DUCT	(22)	(46)	(50)	(49)
HYPERPLASIA, NOS		1 (2%)		
#PANCREATIC ACINUS	(22)	(46)	(50)	(49)
ATROPHY, NOS		2 (4%)		
#STOMACH	(23)	(48)	(50)	(49)
INFLAMMATION, NOS		2 (4%)		
INFLAMMATION, FOCAL		2 (4%)		
PERIARTERITIS			1 (2%)	
HYPERPLASIA, EPITHELIAL		1 (2%)		
#GASTRIC MUCOSA	(23)	(48)	(50)	(49)
HYPERPLASIA, NOS		1 (2%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE C2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 02-0084	LOW DOSE CONTROL (UNTR) 02-0037	LOW DOSE 02-0039	HIGH DOSE 02-0090
#PEYERS PATCH	(23)	(47)	(50)	(49)
HYPERPLASIA, NOS	4 (17%)	6 (13%)		
#COLON	(22)	(43)	(48)	(48)
NEMATODIASIS		3 (7%)		
PARASITISM	2 (9%)			
URINARY SYSTEM				
#KIDNEY	(23)	(49)	(50)	(49)
HYDRONEPHROSIS		1 (2%)		
GLOMERULONEPHRITIS, NOS	4 (17%)	33 (67%)		
INFLAMMATION, INTERSTITIAL		1 (2%)	2 (4%)	
GLOMERULONEPHRITIS, MEMBRANOUS		1 (2%)		
PYELONEPHRITIS, ACUTE	1 (4%)			
INFLAMMATION, CHRONIC		1 (2%)		
PYELONEPHRITIS, CHRONIC	1 (4%)			
PERIARTERITIS			1 (2%)	
NEPHROSIS, NOS	10 (43%)		32 (64%)	22 (45%)
GLOMERULOSCLEROSIS, NOS			1 (2%)	
METAMORPHOSIS FATTY			1 (2%)	
CALCIFICATION, FOCAL	1 (4%)			
#KIDNEY/CORTEX	(23)	(49)	(50)	(49)
CYST, NOS			1 (2%)	
#KIDNEY/TUBULE	(23)	(49)	(50)	(49)
NECROSIS, NOS	1 (4%)			
PIGMENTATION, NOS			1 (2%)	
#URINARY BLADDER	(22)	(41)	(47)	(50)
INFLAMMATION, NOS		1 (2%)		
HYPERPLASIA, EPITHELIAL			1 (2%)	
ENDOCRINE SYSTEM				
#PITUITARY	(21)	(43)	(48)	(43)
HEMORRHAGIC CYST	1 (5%)			
HYPERPLASIA, NOS		2 (5%)		
HYPERPLASIA, FOCAL	1 (5%)			
HYPERPLASIA, CHROMOPHOBE-CELL		1 (2%)		
#ADRENAL	(23)	(46)	(50)	(48)
LIPOIDOSIS			1 (2%)	

\# NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE C2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 02-0084	LOW DOSE CONTROL (UNTR) 02-0037	LOW DOSE 02-0039	HIGH DOSE 02-0090
HYPERTROPHY, NOS			1 (2%)	
#ADRENAL CORTEX	(23)	(46)	(50)	(48)
NODULE		1 (2%)		
HYPERPLASIA, NOS		7 (15%)		
#ADRENAL MEDULLA	(23)	(46)	(50)	(48)
HYPERPLASIA, NOS		4 (9%)		
#THYROID	(21)	(47)	(48)	(49)
HYPERPLASIA, C-CELL	3 (14%)			
HYPERPLASIA, FOLLICULAR-CELL		1 (2%)		
#PANCREATIC ISLETS	(22)	(46)	(50)	(49)
HYPERPLASIA, NOS		1 (2%)		
REPRODUCTIVE SYSTEM				
*MAMMARY GLAND	(23)	(49)	(50)	(50)
GALACTOCELE	1 (4%)	5 (10%)		
CYSTIC DUCTS				1 (2%)
HYPERPLASIA, NOS	1 (4%)	17 (35%)		
HYPERPLASIA, FOCAL				1 (2%)
HYPERPLASIA, PAPILLARY		1 (2%)		
HYPERPLASIA, CYSTIC			1 (2%)	
FIBROCYSTIC DISEASE			1 (2%)	
LACTATION	9 (39%)			
*VAGINA	(23)	(49)	(50)	(50)
HYPERTROPHY, NOS			1 (2%)	
#UTERUS	(23)	(48)	(49)	(45)
DILATATION, NOS				2 (4%)
HYDROMETRA		3 (6%)		
HEMATOMA, NOS			1 (2%)	
INFLAMMATION, SUPPURATIVE		1 (2%)	1 (2%)	
PYOMETRA	3 (13%)			
ABSCESS, NOS		2 (4%)	2 (4%)	
HYPERPLASIA, ADENOMATOUS		5 (10%)		
#CERVIX UTERI	(23)	(48)	(49)	(45)
INFLAMMATION, SUPPURATIVE				1 (2%)
#UTERUS/ENDOMETRIUM	(23)	(48)	(49)	(45)
INFLAMMATION, NOS	1 (4%)	14 (29%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE C2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 02-0084	LOW DOSE CONTROL (UNTR) 02-0037	LOW DOSE 02-0039	HIGH DOSE 02-0090
INFLAMMATION, FOCAL		1 (2%)		
INFLAMMATION, SUPPURATIVE		2 (4%)	8 (16%)	7 (16%)
INFLAMMATION, CHRONIC	1 (4%)			
HYPERPLASIA, NOS	1 (4%)	1 (2%)	1 (2%)	
HYPERPLASIA, CYSTIC	1 (4%)	2 (4%)	4 (8%)	6 (13%)
HYPERPLASIA, ADENOMATOUS		1 (2%)		
#OVARY/OVIDUCT	(23)	(48)	(49)	(45)
INFLAMMATION, NOS		1 (2%)		
INFLAMMATION, ACUTE	1 (4%)			
ABSCESS, NOS	1 (4%)			
#OVARY	(22)	(47)	(50)	(46)
CYST, NOS	3 (14%)	4 (9%)	6 (12%)	
ABSCESS, NOS			1 (2%)	
INFLAMMATION, FOCAL GRANULOMATOU		1 (2%)		
FIBROSIS			2 (4%)	
HYPERPLASIA, INTERSTITIAL CELL		1 (2%)		
NERVOUS SYSTEM				
#BRAIN	(23)	(49)	(50)	(48)
HYDROCEPHALUS, NOS	1 (4%)			
HEMORRHAGE	1 (4%)			
CALCIFICATION, FOCAL	1 (4%)			
SPECIAL SENSE ORGANS				
NONE				
MUSCULOSKELETAL SYSTEM				
NONE				
BODY CAVITIES				
*MEDIASTINUM	(23)	(49)	(50)	(50)
PERIARTERITIS			1 (2%)	
*MESENTERY	(23)	(49)	(50)	(50)
PERIARTERITIS			1 (2%)	
ALL OTHER SYSTEMS				
NONE				

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE C2 (CONCLUDED)

	HIGH DOSE CONTROL (UNTR) 02-0084	LOW DOSE CONTROL (UNTR) 02-0037	LOW DOSE 02-0039	HIGH DOSE 02-0090
SPECIAL MORPHOLOGY SUMMARY				
NO LESION REPORTED			7	7
AUTOLYSIS/NO NECROPSY	2	1		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

APPENDIX D

SUMMARY OF THE INCIDENCE OF NONNEOPLASTIC LESIONS IN MICE TREATED WITH 2,5-TOLUENEDIAMINE SULFATE

TABLE D1
SUMMARY OF THE INCIDENCE OF NONNEOPLASTIC LESIONS IN MALE MICE
TREATED WITH 2,5-TOLUENEDIAMINE SULFATE

	HIGH DOSE CONTROL (UNTR) 05-0077	LOW DOSE CONTROL (UNTR) 05-0037	LOW DOSE 05-0039	HIGH DOSE 05-0089
IMALS INITIALLY IN STUDY	50	50	50	50
IMALS NECROPSIED	46	48	49	49
IMALS EXAMINED HISTOPATHOLOGICALLY**	45	48	48	49
TEGUMENTARY SYSTEM				
SKIN	(46)	(48)	(49)	(49)
EPIDERMAL INCLUSION CYST				1 (2%)
ULCER, FOCAL				1 (2%)
INFLAMMATION, ACUTE/CHRONIC				1 (2%)
INFLAMMATION, CHRONIC FOCAL				1 (2%)
FIBROSIS		1 (2%)		
ALOPECIA		1 (2%)		
HYPERKERATOSIS				1 (2%)
ACANTHOSIS				1 (2%)
SUBCUT TISSUE	(46)	(48)	(49)	(49)
NECROSIS, NOS		1 (2%)		
SPIRATORY SYSTEM				
LUNG/BRONCHUS	(45)	(48)	(47)	(49)
INFLAMMATION, NOS		1 (2%)		
INFLAMMATION, FOCAL		1 (2%)		
LUNG	(45)	(48)	(47)	(49)
BRONCHOPNEUMONIA, NOS			1 (2%)	
INFLAMMATION, NOS		1 (2%)		
INFLAMMATION, INTERSTITIAL		14 (29%)	1 (2%)	
INFLAMMATION, ACUTE FOCAL				1 (2%)
ARTERIOSCLEROSIS, NOS	1 (2%)			
HYPERPLASIA, EPITHELIAL		2 (4%)		
LUNG/ALVEOLI	(45)	(48)	(47)	(49)
HEMORRHAGE				1 (2%)
INFLAMMATION, FOCAL		2 (4%)		
FIBROSIS, FOCAL		1 (2%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
NUMBER OF ANIMALS NECROPSIED
EXCLUDES PARTIALLY AUTOLYZED ANIMALS

TABLE D1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 05-0077	LOW DOSE CONTROL (UNTR) 05-0037	LOW DOSE 05-0039	HIGH DOSE 05-0089
HEMATOPOIETIC SYSTEM				
#SPLEEN	(45)	(47)	(48)	(43)
INFLAMMATION, NOS		1 (2%)		
FIBROSIS	1 (2%)			
HYPERPLASIA, NOS		2 (4%)		
HYPERPLASIA, HEMATOPOIETIC		2 (4%)		
HYPERPLASIA, ERYTHROID		2 (4%)		
HYPERPLASIA, RETICULUM CELL	3 (7%)			
HYPERPLASIA, LYMPHOID		2 (4%)		
HEMATOPOIESIS	1 (2%)			
ERYTHROPOIESIS				2 (5%)
#LYMPH NODE	(35)	(44)	(44)	(36)
HEMORRHAGIC CYST		1 (2%)		
INFLAMMATION, NOS		13 (30%)		
DEGENERATION, CYSTIC		1 (2%)		
HYPERPLASIA, NOS		2 (5%)		
HYPERPLASIA, HEMATOPOIETIC		1 (2%)		
HYPERPLASIA, LYMPHOID		2 (5%)		
MYELOID METAPLASIA		2 (5%)		
#SUBMANDIBULAR L.NODE	(35)	(44)	(44)	(36)
HYPERPLASIA, PLASMA CELL				2 (6%)
#MEDIASTINAL L.NODE	(35)	(44)	(44)	(36)
NECROSIS, NOS		1 (2%)		
#PANCREATIC L.NODE	(35)	(44)	(44)	(36)
INFLAMMATION, NOS		1 (2%)		
HYPERPLASIA, NOS			1 (2%)	
#MESENTERIC L. NODE	(35)	(44)	(44)	(36)
CONGESTION, NOS			3 (7%)	
HEMORRHAGE		1 (2%)		
INFLAMMATION, NOS		9 (20%)		
HYPERPLASIA, NOS			5 (11%)	2 (6%)
#THYMUS	(19)	(34)	(32)	(33)
NECROSIS, NOS		1 (3%)		
CIRCULATORY SYSTEM				
#HEART/VENTRICLE	(44)	(48)	(47)	(49)
MELANIN		2 (4%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE D1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 05-0077	LOW DOSE CONTROL (UNTR) 05-0037	LOW DOSE 05-0039	HIGH DOSE 05-0089
#MYOCARDIUM	(44)	(48)	(47)	(49)
INFLAMMATION, INTERSTITIAL		2 (4%)		
FIBROSIS		5 (10%)		
*BLOOD VESSEL	(46)	(48)	(49)	(49)
INFLAMMATION, NOS		2 (4%)		
*PULMONARY ARTERY	(46)	(48)	(49)	(49)
MINERALIZATION		2 (4%)		
DIGESTIVE SYSTEM				
#SALIVARY GLAND	(43)	(47)	(47)	(45)
INFLAMMATION, NOS		2 (4%)		
PERIVASCULAR CUFFING		1 (2%)		
#LIVER	(45)	(48)	(48)	(49)
HEMATOMA, NOS			1 (2%)	
INFLAMMATION, FOCAL	2 (4%)			
DEGENERATION, NOS	1 (2%)			
NECROSIS, FOCAL		13 (27%)	1 (2%)	
METAMORPHOSIS FATTY	3 (7%)	3 (6%)		
HYPERPLASIA, NODULAR		2 (4%)		
HYPERPLASTIC NODULE				1 (2%)
HYPERPLASIA, FOCAL		1 (2%)		
ANGIECTASIS		1 (2%)		
HEMATOPOIESIS				1 (2%)
MYELOID METAPLASIA		1 (2%)		
#LIVER/PERIPORTAL	(45)	(48)	(48)	(49)
INFLAMMATION, NOS	1 (2%)			
#LIVER/HEPATOCYTES	(45)	(48)	(48)	(49)
DEGENERATION, NOS		1 (2%)		
*GALLBLADDER	(46)	(48)	(49)	(49)
INFLAMMATION, FOCAL		1 (2%)		
*BILE DUCT	(46)	(48)	(49)	(49)
INFLAMMATION, NOS	1 (2%)			
#PANCREAS	(44)	(48)	(45)	(43)
INFLAMMATION, NOS		7 (15%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE D1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 05-0077	LOW DOSE CONTROL (UNTR) 05-0037	LOW DOSE 05-0039	HIGH DOSE 05-0089
INFLAMMATION, FOCAL		1 (2%)		
DEGENERATION, CYSTIC		1 (2%)		
METAMORPHOSIS FATTY		1 (2%)		
#PANCREATIC DUCT	(44)	(48)	(45)	(43)
HYPERPLASIA, NOS		1 (2%)		
#PANCREATIC ACINUS	(44)	(48)	(45)	(43)
HYPERTROPHY, FOCAL		1 (2%)		
HYPERPLASIA, FOCAL		1 (2%)		
#STOMACH	(42)	(47)	(46)	(48)
INFLAMMATION, NOS		13 (28%)		
ULCER, NOS		1 (2%)		
INFLAMMATION, FOCAL		1 (2%)		
INFLAMMATION, INTERSTITIAL		1 (2%)		
PERIARTERITIS				1 (2%)
HYPERPLASIA, NOS		1 (2%)		
HYPERPLASIA, FOCAL	1 (2%)	1 (2%)		
HYPERKERATOSIS		3 (6%)		
ACANTHOSIS		3 (6%)		
#GASTRIC MUCOSA	(42)	(47)	(46)	(48)
HYPERPLASIA, FOCAL		1 (2%)		
#PEYERS PATCH	(43)	(48)	(47)	(48)
HYPERPLASIA, NOS		2 (4%)		
#ILEUM	(43)	(48)	(47)	(48)
HEMORRHAGE		1 (2%)		
INFLAMMATION, NOS		2 (4%)		
#COLON	(38)	(45)	(43)	(46)
PARASITISM		1 (2%)		
URINARY SYSTEM				
#KIDNEY	(45)	(47)	(48)	(48)
CALCULUS, NOS	20 (44%)			
GLOMERULONEPHRITIS, NOS		6 (13%)		
INFLAMMATION, NOS		1 (2%)		
INFLAMMATION, INTERSTITIAL	5 (11%)	23 (49%)		
INFLAMMATION, CHRONIC	1 (2%)			1 (2%)
PERIVASCULITIS	2 (4%)			

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE D-1 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 05-0077	LOW DOSE CONTROL (UNTR) 05-0037	LOW DOSE 05-0039	HIGH DOSE 05-0089
ARTERIOSCLEROSIS, NOS	1 (2%)			
NEPHROSIS, NOS	1 (2%)			
GLOMERULOSCLEROSIS, NOS				1 (2%)
HYPERPLASIA, TUBULAR CELL	2 (4%)			
#KIDNEY/TUBULE	(45)	(47)	(48)	(48)
DEGENERATION, NOS	1 (2%)			
NECROSIS, FOCAL		1 (2%)		
METAMORPHOSIS FATTY	9 (20%)			
#URINARY BLADDER	(44)	(48)	(47)	(47)
INFLAMMATION, NOS		4 (8%)		
HYPERPLASIA, EPITHELIAL		9 (19%)		
ENDOCRINE SYSTEM				
#PITUITARY	(36)	(42)	(41)	(38)
HYPERPLASIA, NOS		3 (7%)		
HYPERPLASIA, FOCAL		3 (7%)		
#ADRENAL CORTEX	(43)	(45)	(45)	(44)
NODULE		1 (2%)		
HYPERTROPHY, FOCAL		1 (2%)		
HYPERPLASIA, NOS		1 (2%)		
#ADRENAL MEDULLA	(43)	(45)	(45)	(44)
DEGENERATION, NOS		1 (2%)		
#THYROID	(40)	(47)	(46)	(46)
LYMPHOCYTIC INFLAMMATORY INFILTR		1 (2%)		
HYPERPLASIA, PAPILLARY		1 (2%)		
HYPERPLASIA, FOLLICULAR-CELL		1 (2%)		
#PANCREATIC ISLETS	(44)	(48)	(45)	(43)
HYPERPLASIA, NOS		2 (4%)		
REPRODUCTIVE SYSTEM				
*PREPUTIAL GLAND	(46)	(48)	(49)	(49)
DILATATION/DUCTS				1 (2%)
INFLAMMATION, SUPPURATIVE			1 (2%)	
ABSCESS, NOS		2 (4%)		
#TESTIS/TUBULE	(45)	(47)	(48)	(48)
DEGENERATION, NOS	2 (4%)	4 (9%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE D1 (CONCLUDED)

	HIGH DOSE CONTROL (UNTR) 05-0077	LOW DOSE CONTROL (UNTR) 05-0037	LOW DOSE 05-0039	HIGH DOSE 05-0089
CALCIFICATION, FOCAL			1 (2%)	
NERVOUS SYSTEM				
#CEREBRAL CORTEX	(45)	(48)	(47)	(44)
MINERALIZATION		3 (6%)		
SPECIAL SENSE ORGANS				
NONE				
MUSCULOSKELETAL SYSTEM				
NONE				
BODY CAVITIES				
*ABDOMINAL CAVITY	(46)	(48)	(49)	(49)
STEATITIS	1 (2%)			
NECROSIS, FAT				1 (2%)
*MESENTERY	(46)	(48)	(49)	(49)
PERIARTERITIS				2 (4%)
ALL OTHER SYSTEMS				
ADIPOSE TISSUE				
NECROSIS, NOS				1
SPECIAL MORPHOLOGY SUMMARY				
NO LESION REPORTED	8		20	17
AUTO/NECROPSY/NO HISTO	1		1	
AUTOLYSIS/NO NECROPSY	4	2	1	1

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE D2
SUMMARY OF THE INCIDENCE OF NONNEOPLASTIC LESIONS IN FEMALE MICE
TREATED WITH 2,5-TOLUENEDIAMINE SULFATE

	HIGH DOSE CONTROL (UNTR) 06-0077	LOW DOSE CONTROL (UNTR) 06-0037	LOW DOSE 06-0039	HIGH DOSE 06-0089
ANIMALS INITIALLY IN STUDY	50	50	50	50
ANIMALS MISSING			1	
ANIMALS NECROPSIED	46	48	45	47
ANIMALS EXAMINED HISTOPATHOLOGICALLY**	46	47	43	46
INTEGUMENTARY SYSTEM				
*SKIN	(46)	(48)	(45)	(47)
FIBROSIS	1 (2%)			
FIBROSIS, FOCAL	1 (2%)			
*SUBCUT TISSUE	(46)	(48)	(45)	(47)
MINERALIZATION		1 (2%)		
FIBROSIS		1 (2%)		
RESPIRATORY SYSTEM				
#LUNG/BRONCHUS	(45)	(46)	(42)	(46)
INFLAMMATION, FOCAL		1 (2%)		
#LUNG	(45)	(46)	(42)	(46)
CONGESTION, NOS			1 (2%)	
INFLAMMATION, INTERSTITIAL	2 (4%)	10 (22%)		
PERIARTERITIS	1 (2%)			
HYPERPLASIA, EPITHELIAL		3 (7%)		
HEMATOPOIETIC SYSTEM				
#BONE MARROW	(44)	(45)	(39)	(41)
MYELOFIBROSIS		1 (2%)		
HYPERPLASIA, HEMATOPOIETIC			1 (3%)	
#SPLEEN	(43)	(46)	(42)	(42)
PLASMA-CELL INFILTRATE			1 (2%)	
HYPERPLASIA, HEMATOPOIETIC		16 (35%)		
HYPERPLASIA, ERYTHROID		6 (13%)		
HYPERPLASIA, RETICULUM CELL	2 (5%)			
HYPERPLASIA, LYMPHOID	4 (9%)	10 (22%)	1 (2%)	

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED
**EXCLUDES PARTIALLY AUTOLYZED ANIMALS

TABLE D2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 06-0077	LOW DOSE CONTROL (UNTR) 06-0037	LOW DOSE 06-0039	HIGH DOSE 06-0089
HEMATOPOIESIS	1 (2%)	1 (2%)	3 (7%)	
ERYTHROPOIESIS			1 (2%)	
MYELOPOIESIS		1 (2%)		
#LYMPH NODE	(41)	(39)	(37)	(32)
CYST, NOS		1 (3%)		
INFLAMMATION, NOS		15 (38%)		
HYPERPLASIA, NOS		1 (3%)		
RETICULOCYTOSIS		1 (3%)		
HYPERPLASIA, HEMATOPOIETIC		2 (5%)		
MYELOID METAPLASIA		1 (3%)		
CIRCULATORY SYSTEM				
#HEART/VENTRICLE	(45)	(46)	(42)	(46)
MELANIN		4 (9%)		
#MYOCARDIUM	(45)	(46)	(42)	(46)
CALCIFICATION, FOCAL	1 (2%)			
*PULMONARY ARTERY	(46)	(48)	(45)	(47)
HYPERPLASIA, NOS	1 (2%)			
DIGESTIVE SYSTEM				
#SALIVARY GLAND	(43)	(45)	(40)	(43)
INFLAMMATION, NOS		2 (4%)		
PERIARTERITIS				1 (2%)
PERIVASCULAR CUFFING		4 (9%)		
#LIVER	(45)	(47)	(42)	(46)
INFLAMMATION, NOS		1 (2%)		
INFLAMMATION, FOCAL	1 (2%)			
NECROSIS, FOCAL		22 (47%)		
CYTOPLASMIC CHANGE, NOS	1 (2%)			
HYPERPLASTIC NODULE		1 (2%)		1 (2%)
HYPERPLASIA, DIFFUSE	1 (2%)			
ANGIECTASIS		1 (2%)		
HEMATOPOIESIS		3 (6%)	2 (5%)	
#LIVER/PERIPORTAL	(45)	(47)	(42)	(46)
INFLAMMATION, NOS	1 (2%)			
*GALLBLADDER	(46)	(48)	(45)	(47)
INFLAMMATION, NOS		3 (6%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE D-2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 06-0077	LOW DOSE CONTROL (UNTR) 06-0037	LOW DOSE 06-0039	HIGH DOSE 06-0089
BILE DUCT	(46)	(48)	(45)	(47)
INFLAMMATION, NOS	1 (2%)	1 (2%)		
PANCREAS	(41)	(44)	(42)	(42)
INFLAMMATION, NOS		5 (11%)		
INFLAMMATION, INTERSTITIAL			1 (2%)	
PERIARTERITIS		1 (2%)		
PANCREATIC DUCT	(41)	(44)	(42)	(42)
LYMPHOCYTIC INFLAMMATORY INFILTR		1 (2%)		
STOMACH	(42)	(44)	(42)	(43)
INFLAMMATION, NOS		7 (16%)		
ULCER, NOS		1 (2%)		
INFLAMMATION, FOCAL		1 (2%)		
HYPERPLASIA, NOS		1 (2%)		
HYPERPLASIA, EPITHELIAL		1 (2%)		
HYPERPLASIA, ADENOMATOUS		1 (2%)		
HYPERKERATOSIS		1 (2%)		
ACANTHOSIS		1 (2%)		
GASTRIC MUCOSA	(42)	(44)	(42)	(43)
HYPERPLASIA, FOCAL		1 (2%)		
PEYERS PATCH	(43)	(44)	(42)	(42)
HYPERPLASIA, NOS		1 (2%)		
INARY SYSTEM				
KIDNEY	(43)	(46)	(43)	(45)
CONGESTION, NOS			1 (2%)	
GLOMERULONEPHRITIS, NOS		14 (30%)		
INFLAMMATION, INTERSTITIAL	3 (7%)	16 (35%)		
GLOMERULONEPHRITIS, CHRONIC				1 (2%)
PERIVASCULITIS	4 (9%)			
KIDNEY/GLOMERULUS	(43)	(46)	(43)	(45)
AMYLOIDOSIS	1 (2%)			
KIDNEY/PELVIS	(43)	(46)	(43)	(45)
INFLAMMATION, ACUTE/CHRONIC	1 (2%)			
URINARY BLADDER	(41)	(46)	(34)	(41)
INFLAMMATION, NOS		4 (9%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
NUMBER OF ANIMALS NECROPSIED

TABLE D2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 06-0077	LOW DOSE CONTROL (UNTR) 06-0037	LOW DOSE 06-0039	HIGH DOSE 06-0089
HYPERPLASIA, EPITHELIAL		10 (22%)		
#U.BLADDER/SUBMUCOSA	(41)	(46)	(34)	(41)
INFLAMMATION, CHRONIC				1 (2%)
ENDOCRINE SYSTEM				
#PITUITARY	(37)	(42)	(38)	(38)
HYPERPLASIA, FOCAL		6 (14%)		
#ADRENAL CORTEX	(43)	(45)	(38)	(41)
NODULE		3 (7%)		
#THYROID	(30)	(43)	(36)	(34)
FOLLICULAR CYST, NOS		1 (2%)		
INFLAMMATION, NOS		1 (2%)		
INFLAMMATION, CHRONIC FOCAL				1 (3%)
REPRODUCTIVE SYSTEM				
*MAMMARY GLAND	(46)	(48)	(45)	(47)
GALACTOCELE		1 (2%)		
HYPERPLASIA, NOS		4 (8%)		
#UTERUS	(43)	(45)	(41)	(42)
HYDROMETRA	4 (9%)	1 (2%)	2 (5%)	2 (5%)
INFLAMMATION, ACUTE			1 (2%)	1 (2%)
ABSCESS, NOS		3 (7%)		
FIBROSIS		1 (2%)		
#UTERUS/ENDOMETRIUM	(43)	(45)	(41)	(42)
DILATATION, NOS			5 (12%)	
CYST, NOS	2 (5%)			
INFLAMMATION, NOS		10 (22%)		
INFLAMMATION, SUPPURATIVE		4 (9%)	1 (2%)	4 (10%)
INFLAMMATION, ACUTE			1 (2%)	
INFLAMMATION, ACUTE SUPPURATIVE			4 (10%)	
HYPERPLASIA, NOS	1 (2%)	4 (9%)		
HYPERPLASIA, CYSTIC	35 (81%)	18 (40%)	18 (44%)	28 (67%)
HYPERPLASIA, ADENOMATOUS		1 (2%)		
#OVARY/OVIDUCT	(43)	(45)	(41)	(42)
INFLAMMATION, NOS		5 (11%)		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

TABLE D2 (CONTINUED)

	HIGH DOSE CONTROL (UNTR) 06-0077	LOW DOSE CONTROL (UNTR) 06-0037	LOW DOSE 06-0039	HIGH DOSE 06-0089
INFLAMMATION, SUPPURATIVE			2 (5%)	
OVARY	(41)	(45)	(36)	(39)
CYST, NOS	1 (2%)	3 (7%)		4 (10%)
INFLAMMATION, NOS		4 (9%)		
LYMPHOCYTIC INFLAMMATORY INFILTR		1 (2%)		
INFLAMMATION, SUPPURATIVE		10 (22%)	2 (6%)	6 (15%)
INFLAMMATION, ACUTE			1 (3%)	
INFLAMMATION, ACUTE SUPPURATIVE			1 (3%)	
ABSCESS, NOS		4 (9%)		
INFLAMMATION, CHRONIC			4 (11%)	
DEGENERATION, CYSTIC		1 (2%)		
ATROPHY, NOS			1 (3%)	
OVARY/FOLLICLE	(41)	(45)	(36)	(39)
MULTIPLE CYSTS			1 (3%)	
RVOUS SYSTEM				
NONE				
ECIAL SENSE ORGANS				
NONE				
SCULOSKELETAL SYSTEM				
BONE	(46)	(48)	(45)	(47)
RESORPTION		3 (6%)		
VERTEBRA	(46)	(48)	(45)	(47)
OSTEOSCLEROSIS	1 (2%)			
DY CAVITIES				
NONE				
L OTHER SYSTEMS				
OMENTUM				
NECROSIS, FAT		1		

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
NUMBER OF ANIMALS NECROPSIED

TABLE D-2 (CONCLUDED)

	HIGH DOSE CONTROL (UNTR) 06-0077	LOW DOSE CONTROL (UNTR) 06-0037	LOW DOSE 06-0039	HIGH DOSE 06-0089
SPECIAL MORPHOLOGY SUMMARY				
NO LESION REPORTED	1	1	4	5
ANIMAL MISSING/NO NECROPSY			1	
AUTO/NECROPSY/HISTO PERF	2	2		1
AUTO/NECROPSY/NO HISTO		1	2	1
AUTOLYSIS/NO NECROPSY	4	2	4	3

NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

Review of the Bioassay of 2,5-Toluenediamine Sulfate*
for Carcinogenicity
by the Data Evaluation/Risk Assessment Subgroup
of the Clearinghouse on Environmental Carcinogens

June 29, 1978

The Clearinghouse on Environmental Carcinogens was established in May, 1976, in compliance with DHEW Committee Regulations and the Provisions of the Federal Advisory Committee Act. The purpose of the Clearinghouse is to advise the Director of the National Cancer Institute (NCI) on its bioassay program to identify and to evaluate chemical carcinogens in the environment to which humans may be exposed. The members of the Clearinghouse have been drawn from academia, industry, organized labor, public interest groups, State health officials, and quasi-public health and research organizations. Members have been selected on the basis of their experience in carcinogenesis or related fields and, collectively, provide expertise in chemistry, biochemistry, biostatistics, toxicology, pathology, and epidemiology. Representatives of various Governmental agencies participate as ad hoc members. The Data Evaluation/Risk Assessment Subgroup of the Clearinghouse is charged with the responsibility of providing a peer review of reports prepared on NCI-sponsored bioassays of chemicals studied for carcinogenicity. It is in this context that the below critique is given on the bioassay of 2,5-Toluenediamine Sulfate for carcinogenicity.

Although a carcinogenic response was not demonstrated, the reviewer said that the evidence was suggestive that the compound may have a carcinogenic potential. He recommended that it be considered for retest. In his critique, he noted several experimental flaws, including the use of animals from different shipments, the conduct of the subchronic study in a different mouse strain than used in the chronic phase, and the start of the high dose rats on test some months after the initiation of the low dose animal group. The reviewer said the compound warranted further testing because of the experimental design and study conduct deficiencies, as well as the fact that 2,5-Toluenediamine Sulfate

had been shown to be positive in the Ames assay. The reviewer moved that the report on the bioassay of 2,5-Toluenediamine Sulfate be accepted as written but that the compound be considered for retest. The motion was approved without objection.

Clearinghouse Members present:

Arnold L. Brown (Chairman), Mayo Clinic
Paul Nettesheim, National Institute of Environmental Health Sciences
Verne Ray, Pfizer Medical Research Laboratory
Verald K. Rowe, Dow Chemical U.S.A.
Michael B. Shimkin, University of California at San Diego
Louise Strong, University of Texas Health Sciences Center

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* Subsequent to this review, changes may have been made in the bioassay report either as a result of the review or other reasons. Thus, certain comments and criticisms reflected in the review may no longer be appropriate.

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EXHIBIT F

Fd Cosmet. Toxicol. Vol. 15. pp. 447-451. Pergamon Press 1977. Printed in Great Britain

TERATOGENICITY OF 2,5-DIAMINOTOLUENE, A HAIR-DYE CONSTITUENT, IN MICE

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(*Received 21 December 1976*)

Summary—Pregnant mice were injected with 2,5-diaminotoluene dihydrochloride, a hair-dye constituent, in a single sc dose of 50 mg/kg body weight on various days of pregnancy, and the foetuses were examined for abnormalities on day 18 of pregnancy. A low incidence of exencephaly and prosoposchisis and a high incidence of skeletal malformations were found in the group treated on day 8. Similar malformations were also detected in two additional groups treated with a higher sc dose (75 mg/kg) or with an ip dose of 50 mg/kg on day 8 of pregnancy. No such malformed foetuses were found in the untreated control group or in the groups treated on days 10–14 of pregnancy, and only a very low incidence of vertebral and rib anomalies followed treatment on day 7 or 9.

Introduction

In recent years, there has been growing concern over the safety of hair dyes. Ames, Kammen & Yamasaki (1975) demonstrated that some hair-dye constituents were mutagenic in bacterial test systems. Searle, Harnden, Venitt & Gyde (1975) also reported that certain hair colourants were mutagenic in bacteria, and the results of their tests in mice indicated some carcinogenic activity. It has been reported that the appearance of dark urine has followed the human use of hair dyes (Marshall & Palmer, 1973; Peter, 1975); since such dyes are used mainly by women, and sometimes during pregnancy, teratogenicity tests of hair dyes should be required. Wernick, Lanman & Fraux (1975) studied the effects of semipermanent-type hair dyes on reproduction and foetal development in rats and rabbits but could detect no evidence of teratogenic effect. Burnett, Goldenthal, Harris, Wazeter, Strausburg, Kapp & Voelker (1976) also reported that five oxidation hair-dye formulations tested were not teratogenic in rats. However, there is too little information in their experiments on several dozen animals to permit conclusions to be drawn about the safety of hair dyes to the millions of human foetuses.

This report concerns the teratogenicity of 2,5-diaminotoluene, which is an essential constituent of conventional oxidative-type hair dyes. This chemical was previously found to be mutagenic in the TA1538 strain of *Salmonella typhimurium* (Ames *et al.* 1975) and has been shown to penetrate human skin during hair dyeing (Kiese & Rauscher, 1968).

Experimental

Material. A hair-dye constituent, 2,5-diaminotoluene dihydrochloride, was obtained from Tokyo Kasei Kogyo Co. Ltd., Tokyo. Assay by gas chromatography showed this material to be 99·9% pure.

Animals and dosage. The animals used were supplied by Nippon CLEA Co. Ltd., Tokyo, from a closed colony of JCL:ddN mice. They were housed at 23 ± 1°C and 55 ± 5% humidity and were given a solid diet of NMF, produced by Oriental Yeast Co. Ltd., Tokyo, and tap-water *ad lib.* Primigravid females, 10 wk or older and weighing 25–30 g, were caged with males of the same strain in pairs overnight, and the next morning females with vaginal plugs were considered to be in day 0 of pregnancy. The pregnant females were divided into eight groups, each of ten or 11 mice, and injected on one of days 7–14 of pregnancy with 2,5-diaminotoluene dihydrochloride, dissolved in distilled water, in a single sc dose of 50 mg/kg (calculated on the treatment-day weight), the maximum tolerated dose for pregnant mice. An untreated control group (13 females) was also maintained. Since the results indicated that 50 mg/kg given on day 8 had the greatest teratogenic effect, the study was extended to include a higher dose of 2,5-diaminotoluene and a different route of administration, an additional two groups of females (ten and 12 mice, respectively) being given either 75 mg/kg sc or an ip dose of 50 mg/kg on day 8 of pregnancy. All mice were killed under ether anaesthesia on day 18 of pregnancy and the uterus was examined for resorption sites. Surviving foetuses were counted, removed, examined for external malformations under a dissecting microscope, weighed and fixed in 95% ethanol. They were then cleared in 1% KOH and stained with Alizarin Red S for demonstration of skeletal abnormalities.

Results and Discussion

The results are summarized in Tables 1 & 2. A fairly low incidence of cranio-facial malformations such as exencephaly and prosoposchisis was found in foetuses from dams treated sc or ip with 2,5-diaminotoluene dihydrochloride on day 8 of pregnancy (Fig. 1a, b). No such cranio-facial malformations have been detected in foetuses of untreated ddN-strain mice examined in this laboratory in the last 8 yr. A high incidence of skeletal malformations, principally fused or distorted thoracic vertebrae associated with absent or fused ribs, was also observed in foetuses

Table 1. *Reproduction and teratology data for mice treated with 2,5-diaminotoluene dihydrochloride in a single sc injection of 50 mg/kg body weight on days 7–14 of pregnancy*

Time of treatment (day of pregnancy)	No. of dams/group	Total no. of: Implantations	Total no. of: Dead foetuses or resorptions*	Total no. of: Live foetuses	Foetal weight† (g)	No. of: Ossified vertebrae†	No. of: Malformed foetuses‡	No. of foetuses with: AH	E	VRM	P
7	11	126	16 (13)	110	1·3 ± 0·13	36 ± 1·3	3 (3)	1		2	
8	11	119	10 (8)	109	1·3 ± 0·14	36 ± 1·0	20 (18)		3	15	2
9	10	108	10 (9)	98	1·3 ± 0·09	36 ± 1·3	1 (1)			1	
10	10	100	7 (7)	93	1·3 ± 0·18	36 ± 1·6	0				
11	10	105	11 (11)	94	1·4 ± 0·16	36 ± 1·6	0				
12	10	95	11 (12)	84	1·3 ± 0·13	36 ± 1·3	0				
13	10	112	10 (9)	102	1·3 ± 0·14	36 ± 1·5	1 (1)	1			
14	10	89	6 (7)	83	1·3 ± 0·17	36 ± 1·5	0				
Control	13	144	12 (8)	132	1·3 ± 0·12	36 ± 1·2	0				

AH = Abdominal hernia E = Exencephaly VRM = Vertebral and rib malformation P = Prosoposchisis

*Expressed in parentheses as percentage of total implantations.

†Means ± SD.

‡Expressed in parentheses as percentage of live foetuses.

Table 2. *Reproduction and teratology data for mice treated with a single sc or ip dose of 2,5-diaminotoluene dihydrochloride on day 8 of pregnancy*

Dose (mg/kg) and route	No. of dams/group	No. of maternal deaths	Total no. of: Implantations	Total no. of: Dead foetuses or resorptions*	Total no. of: Live foetuses	Foetal weight† (g)	No. of: Ossified vertebrae†	No. of: Malformed foetuses	No. of foetuses with: E	VRM
75, sc	10	6	34	14 (41)	20	1·4 ± 0·09	36 ± 1·2	7 (35)	1	6
50, ip	12	4	78	40 (51)	38	1·3 ± 0·12	35 ± 0·8	17 (45)	1	16

E = Exencephaly VRM = Vertebral and rib malformations

*Expressed in parentheses as percentage of total implantations.

†Means ± SD.

‡Expressed in parentheses as percentage of live foetuses.

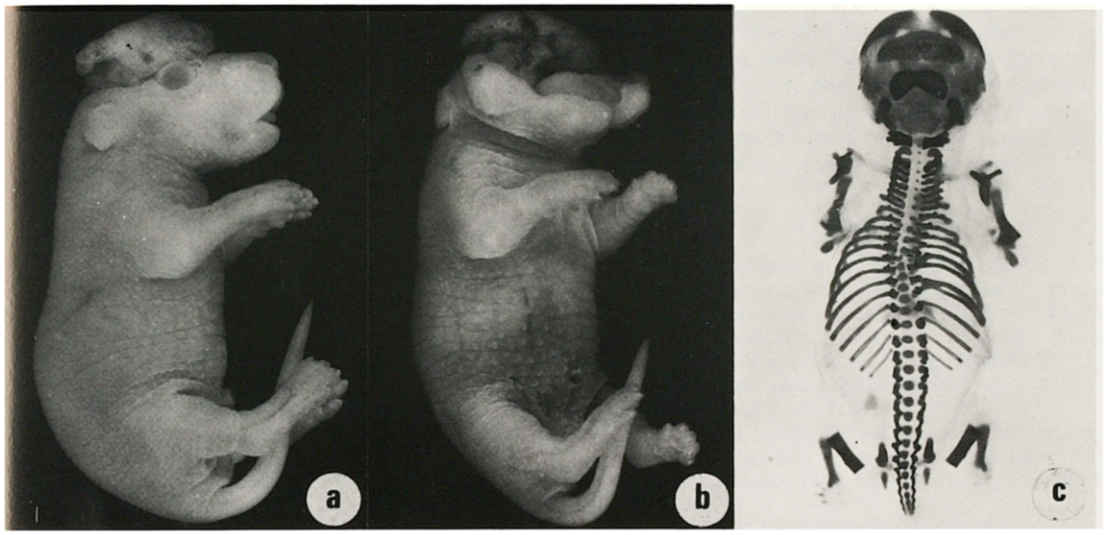

Fig. 1. Malformed foetuses from pregnant mice treated sc with 50 mg 2,5-diaminotoluene dihydrochloride/kg on day 8 of pregnancy, showing (a) exencephaly, (b) prosoposchisis and (c) distorted vertebrae associated with fused ribs.

treated on day 8 of pregnancy (Fig. 1c). Similar vertebral and rib abnormalities were found in a few foetuses in the groups treated on day 7 or 9 of pregnancy. There were no statistically significant differences in the incidence of intrauterine deaths among the groups treated sc with 50 mg/kg on various days of pregnancy or between these and the untreated group, but in groups treated sc with a dose of 75 mg/kg or ip with 50 mg/kg on day 8, some dams died and nearly half of the foetuses died or were resorbed. There were no significant differences in either mean foetal weight or mean number of ossified vertebrae in all eleven treated and untreated groups.

These results suggest that 2,5-diaminotoluene is markedly teratogenic in mice. There were no apparent differences in the types of malformation induced by different doses or by different routes of administration in this series of experiments. The mechanism of this teratogenicity is obscure, and although the 50-mg/kg sc dose was not overtly toxic to the dams, the possibility that it exerted some maternal toxicity and thus affected foetal development indirectly, cannot be altogether dismissed. Nevertheless, the results of Ames *et al.* (1975), showing that 2,5-diaminotoluene was mutagenic in bacteria, suggested that the chemical had the ability to damage cellular DNA, and it is possible, therefore, that the DNA of embryonic cells is involved in the findings reported here. As Connors (1975) reported, numerous DNA-damaging chemicals, such as alkylating agents and electrophilic reactants, have been shown to be teratogenic in mammals, including man in some cases.

Burnett *et al.* (1976) studied five hair-dye formulations containing 2,5-diaminotoluene and found them to be non-teratogenic in rats at dose levels comparable with human exposure. In the present experiment, however, the teratogenicity of 2,5-diaminotoluene was demonstrated at the maximum tolerated dose (50 mg/kg sc) and roughly median lethal doses (75 mg/kg sc and 50 mg/kg ip) for pregnant mice. As Epstein (1973) stated, agents should be tested at dose levels higher than might be anticipated in man following high-level accidental exposure. This is an essential step towards counteracting the insensitivity of conventional test systems, which are based on very small numbers of animals compared with the millions of humans possibly at risk. Kiese & Rauscher (1968) reported that less than 0·2%, or about 4·6 mg, 2,5-diaminotoluene was absorbed through human skin during a standard hair-dyeing procedure. The dose for a 50-kg woman may therefore be calculated as roughly 0·1 mg/kg or 500 times less than the dose (50 mg/kg) shown to be teratogenic in the present experiment. Occasionally, however, human subjects are more sensitive to certain chemicals than are experimental animals. For instance, humans are 60 times more sensitive than mice and 700 times more sensitive than hamsters to thalidomide teratogenicity (Epstein, 1973). Thus, a compound to which man is exposed at levels far lower than the dose that is teratogenic in experimental animals cannot be considered necessarily non-teratogenic in man. The finding that 2,5-diaminotoluene, one of the hair-dye constituents, is teratogenic in mice raises the question, therefore, as to whether there is any risk to the human foetus from maternal use of hair dyes during pregnancy and strongly suggests the need for comprehensive studies on the effect of hair dyes on reproduction.

REFERENCES

Ames, B. N., Kammen, H. O. & Yamasaki, E. (1975). Hair dyes are mutagenic: Identification of a variety of mutagenic ingredients. *Proc. natn. Acad. Sci. U.S.A.* **72,** 2423.

Burnett, C., Goldenthal, E. I., Harris, S. B., Wazeter, F. X., Strausburg, J., Kapp, R. & Voelker, R. (1976). Teratology and percutaneous toxicity studies on hair dyes. *J. Toxicol. envir. Hlth* **1,** 1027.

Connors, T. A. (1975). Cytotoxic agents in teratogenic research. In *Teratology Trends and Applications.* Edited by C. L. Berry and D. E. Poswillo. p. 49. Springer-Verlag, Berlin.

Epstein, S. S. (1973). Environment and teratogenesis. In *Pathobiology of Development—Or Ontogeny Revisited.* Edited by E. V. D. Perrin and M. J. Finegold. p. 105. The Williams & Wilkins Company, Baltimore.

Kiese, M. & Rauscher, E. (1968). The absorption of *p*-toluenediamine through human skin in hair dyeing. *Toxic. appl. Pharmac.* **13,** 325.

Marshall, S. & Palmer, W. S. (1973). Dark urine after hair coloring. *J. Am. med. Ass.* **226,** 1010.

Peter, J. B. (1975). Pigmenturia from "Loving Care" hair dye. *New Engl. J. Med.* **293,** 458.

Searle, C. E., Harnden, D. G., Venitt, S. & Gyde, O. H. B. (1975). Carcinogenicity and mutagenicity tests of some hair colourants and constituents. *Nature, Lond.* **255,** 506.

Wernick, T., Lanman, B. M. & Fraux, J. L. (1975). Chronic toxicity, teratologic, and reproduction studies with hair dyes. *Toxic. appl. Pharmac.* **32,** 450.

EXHIBIT G

Environmental Health Perspectives
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Formation and Persistence of Arylamine DNA Adducts *In Vivo*

by Frederick A. Beland* and Fred F. Kadlubar*

Aromatic amines are urinary bladder carcinogens in man and induce tumors at a number of sites in experimental animals including the liver, mammary gland, intestine, and bladder. In this review, the particular pathways involved in the metabolic activation of aromatic amines are considered as well as the specific DNA adducts formed in target and nontarget tissue. Particular emphasis is placed on the following compounds: 1-naphthylamine, 2-naphthylamine, 4-aminobiphenyl, 4-acetylaminobiphenyl, 4-acetylamino-4′-fluorobiphenyl, 3,2′-dimethyl-4-aminobiphenyl, 2-acetylaminofluorene, benzidine, *N*-methyl-4-aminoazobenzene, 4-aminoazobenzene, and 2-acetylaminophenanthrene.

Introduction

Aromatic amines and amides were originally associated with the induction of bladder tumors in man and have subsequently been found to be carcinogenic at a number of sites in a variety of experimental animals (*1,2*). The initial step in the metabolic activation of these compounds usually involves an *N*-oxidation (Fig. 1), which can be catalyzed by cytochrome P-450, the flavin-containing monooxygenase (FMO), or peroxidases, such as prostaglandin H synthase (PHS). Primary arylamines are *N*-oxidized to *N*-hydroxy arylamines that can undergo an acid-catalyzed reaction with DNA or can be further metabolized to a reactive electrophile through acetyl coenzyme A (AcCoA)-dependent *O*-acetylation, ATP-dependent *O*-aminoacylation, or 3′-phosphoadenosine 5′-phosphosulfate (PAPS)-dependent *O*-sulfonylation. The *N*-oxidation of secondary arylamines results in *N*-hydroxy metabolites that do not react directly with DNA but are further converted into electrophilic derivatives by *O*-sulfonylation.

Aromatic amides are *N*-oxidized only by the cytochrome P-450 monooxygenases, and since the *N*-hydroxy arylamides (arylhydroxamic acids) that are formed

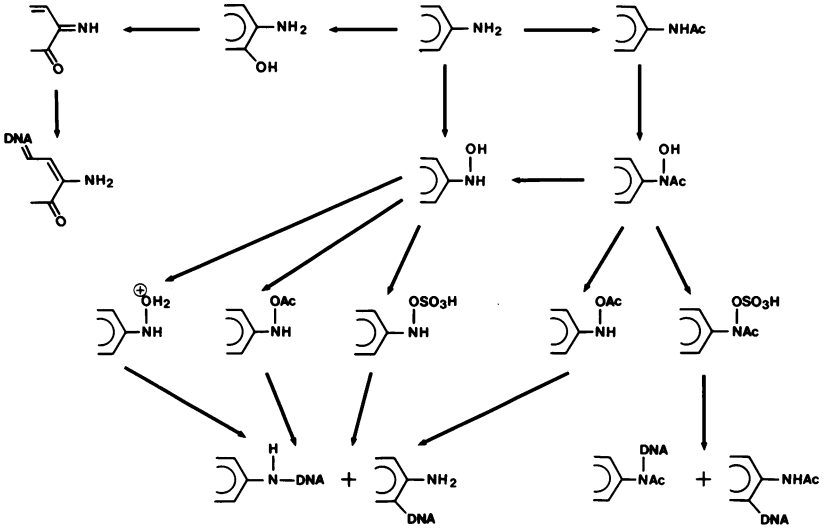

FIGURE 1. Metabolic pathways leading to DNA adducts from arylamines and arylamides.

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are not reactive, further metabolic activation is required. This can be accomplished by at least three pathways: *O*-sulfonylation, deacetylation to a *N*-hydroxy arylamine, or intramolecular *N,O*-acyltransfer to a *N*-acetoxy arylamine. In this review, we consider how these various metabolic pathways lead to the specific arylamine and arylamide DNA adducts that have been detected *in vivo*. We also discuss the relationship between the persistence of specific adducts and tumor induction.

1-Naphthylamine

Although 1-naphthylamine (1-NA) has not been found to be carcinogenic in any bioassay (*1,3,4*), its *N*-oxidized derivative, *N*-hydroxy-1-NA, is strongly carcinogenic at sites of application (*5–7*). The *in vitro* reaction of *N*-hydroxy-1-NA with DNA yields at least three adducts (Fig. 2): *N*-(deoxyguanosin-O^6-yl)-1-NA, 2-(deoxyguanosin-O^6-yl)-1-NA, and a decomposition product of the latter adduct (*8*). *N*-(Guan-8-yl)-1-NA has also been isolated after acidic hydrolyis of *N*-hydroxy-1-NA-treated DNA (*9*) but has not been detected after enzymatic hydrolysis (Kadlubar, unpublished observation). As with other primary *N*-hydroxy arylamines, DNA adduct formation with *N*-hydroxy-1-NA is acid-catalyzed and is 20-fold greater at pH 5 as compared to pH 7.

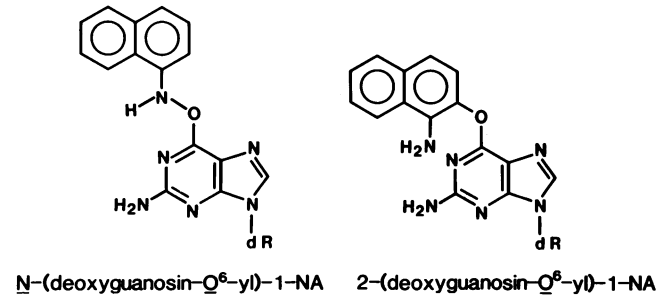

FIGURE 2. DNA adducts derived from 1-naphthylamine.

The DNA adducts formed at the subcutaneous injection site of rats administered *N*-hydroxy-1-NA have recently been examined (*7*). *N*-(Deoxyguanosin-O^6-yl)-1-NA was the major adduct detected 1 day after treatment and the total binding decreased by only 30% during the next 6 days. *N*-Hydroxy-1-NA bound to the DNA 20-fold better than the isomeric *N*-hydroxy-2-NA, which was consistent with the relative carcinogenicity of the two compounds. Furthermore, alternating the administration of both *N*-hydroxy arylamines decreased both the incidence of *N*-hydroxy-1-NA-induced tumors and the persistence of the adducts resulting from this compound.

Dogs have been used extensively as a model for arylamine carcinogenesis in man. Therefore, as part of a study relating the extent of adduct formation to carcinogenic potency, the binding of 1-NA to hepatic and urothelial DNA of dogs was assessed (*10,11*). Adduct formation from 1-NA was not detected in urothelial DNA and only very low levels were found in hepatic DNA. The failure to detect significant binding could indicate very efficient DNA repair processes or, alternatively, may indicate that 1-NA cannot be activated *in vivo*. The latter possibility appears more likely because in a recent study (*12*), cytochromes P-450 and FMO from a number of species were unable to catalyze the oxidation of 1-NA to *N*-hydroxy-1-NA.

2-Naphthylamine

In contrast to 1-NA, its isomer 2-NA is carcinogenic in a number of species including rats, dogs, and humans (*1*). When the *N*-oxidized derivative, *N*-hydroxy-2-NA, was reacted *in vitro* at pH 5 with DNA, three adducts were detected (Fig. 3): an imidazole ring-opened derivative of *N*-(deoxyguanosin-8-yl)-2-NA(50%), 1-(deoxyguanosin-N^2-yl)-2-NA (30%)), and 1-(deoxyadenosin-N^6-yl)-2-NA (15%) (*13*). Although 2-NA is carcinogenic and 1-NA is not, it should be noted that the *N*-hydroxy derivative of 2-NA does not bind to DNA *in vitro* nearly as well as does *N*-hydroxy-1-NA (*14*). This difference in binding also occurs *in vivo* because, as mentioned above, *N*-hydroxy-2-NA bound to injection site DNA in rats to a much lower extent than was found with *N*-hydroxy-1-NA (*7*). This reduced binding was probably responsible for the small tumor yield being recorded with *N*-hydroxy-2-NA.

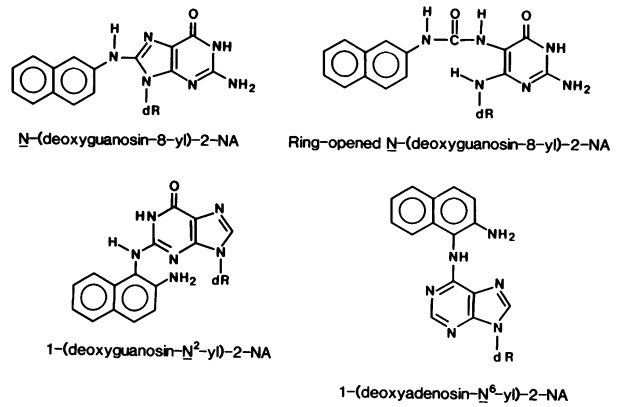

FIGURE 3. DNA adducts derived from 2-naphthylamine.

The DNA adduct derived from 2-NA in target (urothelium) and nontarget (liver) tissues of dogs have also been reported (*10,11*). Two days following the oral administration of 2-NA, the same three adducts that were found *in vitro* from *N*-hydroxy-2-NA were detected in both tissues with 4-fold higher binding levels being observed in the urothelial DNA. The major product in both tissues was the ring-opened derivative of *N*-(deoxyguanosin-8-yl)-2-NA, followed by lower levels of 1-(deoxyadenosin-N^6-yl)-2-NA and 1-(deoxyguanosin-N^2-yl)-2-NA. Although there is not an exact correspondence between the ratios of adducts found *in vivo* and *in vitro*, the commonality of adducts suggests that *N*-hy-

droxy-2-NA could be the ultimate carcinogenic species *in vivo*. This view is strengthened by a pharmacokinetic model which correctly predicts bladder tumor susceptibility in different species as a function of urine pH, voiding interval and resorption (*15*).

When the extent of binding was measured 7 days after 2-NA administration, the N^2-deoxyguanosine adduct appeared to persist in the dog liver, while both this adduct and the C8-deoxyguanosine adduct persisted in the bladder(*10,11*). The differential loss of adducts indicates that active repair processes are present in both tissues, and the relative persistence of the C8-deoxyguanosine adduct in target but not in the nontarget tissue suggests that this adduct may be a critical lesion for the initiation of urinary bladder tumors.

Peroxidative enzymes, such as PHS, have been shown to catalyze both the *N*- and ring-oxidation of 2-NA (*16*). When DNA was included in these *in vitro* incubations, six adducts were detected (*17*), three arising from *N*-hydroxy-2-NA, while the remaining three appeared to be formed from 2-imino-1-naphthoquinone, the oxidative product of 2-amino-1-naphthol (Fig. 4). Since PHS activity has been demonstrated in the dog bladder (*18*), additional experiments were conducted to determine if 2-amino-1-naphthol-derived adducts could be detected *in vivo* (*17*). Two days following the administration of 2-NA, adducts that accounted for about 20% of the total binding and that were indicative of 2-imino-1-naphthoquinone formation were identified in urothelial but not liver DNA. Thus, it appears that PHS may have a significant role in arylamine bladder carcinogenesis.

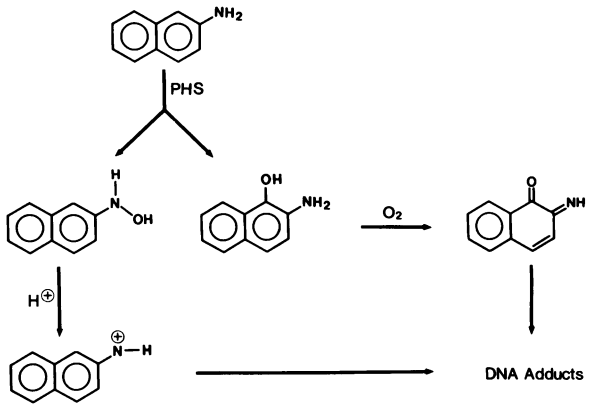

FIGURE 4. Metabolic activation of 2-naphthylamine by prostaglandin H synthase.

4-Aminobiphenyl and 4-Acetylaminobiphenyl

The arylamide, 4-acetylaminobiphenyl (AABP), differs from a number of aromatic amides in that its primary target in rats is the mammary gland as opposed to the liver (*19*). Although the adducts in the mammary gland have not been characterized, Kriek (*20*) has compared the binding of AABP to hepatic DNA with that of the strong hepatocarcinogen 2-acetylaminofluorene (AAF) in order to determine why AABP is such a poor inducer of liver tumors. Following administration of a single dose of the presumed proximate carcinogen, *N*-hydroxy-AABP, the total binding to hepatic DNA was about 25% of that found with the hydroxamic acid of AAF. When the individual adducts were examined, approximately 20% retained their acetyl groups and thus presumably arose from a sulfotransferase-catalyzed intermediate, while the remaining 80% were nonacetylated and may have resulted from deacetylation, *N,O*-acetyltransfer (*21*), or deacetylation followed by acetyl coenzyme A-dependent *O*-acetylation (*22*). Although the nonacetylated adducts were not characterized, the acetylated adducts were subsequently identified (*23*) as 3-(deoxyguanosin-N^2-yl)-AABP and *N*-(deoxyguanosin-8-yl)-AABP (Fig. 5).

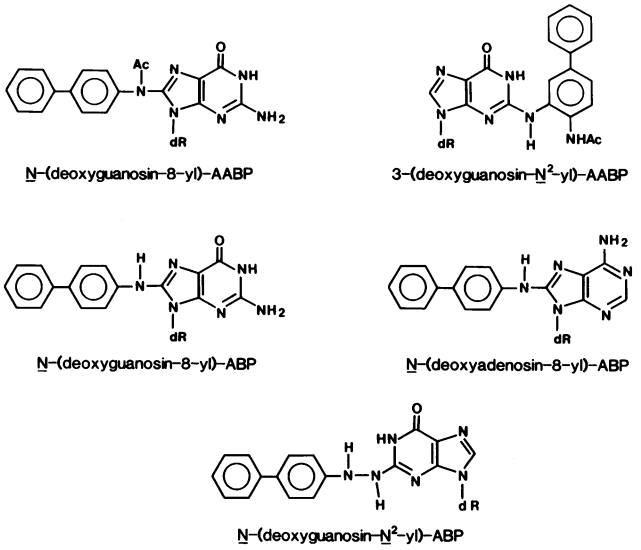

FIGURE 5. DNA adducts derived from 4-acetylaminobiphenyl and 4-aminobiphenyl.

Recently Gupta and Dighe (*24*) have used a ^{32}P-postlabeling technique to examine the types and persistence of adducts following the IP administration of several arylhydroxamic acids to rats. Maximum binding of *N*-hydroxy-AABP to hepatic DNA was observed 30 min after treatment but the value was only about 10% of the maximum value found with a similar dose of *N*-hydroxy-AAF. Initially, the binding rapidly decreased, by 50% in 4 hr and 75% in 24 hr, and then more slowly, resulting in approximately 10% of the adducts persisting after 29 days. The major adduct (ca. 70%) was a nonacetylated product which coeluted with a *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (ABP) standard (Fig. 5), while an additional nonacetylated adduct accounted for 7% of the initial binding. Two acetylated adducts were also detected; *N*-(deoxyguanosin-8-yl)-AABP was the major acetylated product (13%), while an adduct tentatively identified as 3-(deoxyguanosin-N^2-yl)-AABP was found in smaller amounts. After 29 days, the only adducts that were detected were the two nonacetylated ones as well as 3-(deoxyguanosin-N^2-yl)-AABP.

Among the aromatic amine carcinogens, ABP is the most potent bladder carcinogen in dogs (*25*). Since dogs are deficient in *N*-acetylases (*26,27*), *N*-hydroxy arylamines have been proposed to be the ultimate carcinogenic form in this species. When *N*-hydroxy-ABP was reacted with calf thymus DNA at pH 5, three adducts resulted (Fig. 5): *N*-(deoxyguanosin-8-yl)-ABP (80%), *N*-(deoxyadenosin-8-yl)-ABP (15%), and *N*-(deoxyguanosin-N^2-yl)ABP (5%) (*11,28*). The deoxyadenosine adduct was unusual in that it was substituted at C8, while the N^2-deoxyguanosine adduct was interesting because it contained a hydrazo linkage. When dogs were administered ABP a similar ratio of adducts (*11,28*) was found with *N*-(deoxyguanosin-8-yl)-ABP being the major product (76%), followed by *N*-(deoxyguanosin-N^2-yl)-ABP (15%), and then *N*-(deoxyadenosin-8-yl)-ABP (9%). Binding to urothelial DNA increased between 1 and 2 days, and appeared to remain constant for an additional 5 days. ABP gave the most extensive binding of the arylamine carcinogens examined, which correlated with it being the strongest carcinogen. However, essentially the same level of binding, adduct distribution and persistence were found in the liver, which is not a target for ABP.

4-Acetylamino-4′-fluorobiphenyl

Substitution of AABP with a 4′-fluoro group results in a compound that induces renal as well as hepatic and mammary gland tumors in rats (*29–31*). Kriek and Hengeveld (*32*) have examined the DNA adducts present in both liver and kidney in rats administered a single dose of the arylhydroxamic acid, *N*-hydroxy-4-acetylamino-4′-fluorobiphenyl (AAFBP). A similar level of binding was found in both tissues with the majority (80%) of the adducts being nonacetylated. The nonacetylated adducts were not characterized but were suggested to be decomposition products of *N*-(deoxyguanosin-8-yl)-4-amino-4′-fluorobiphenyl (AFBP) (Fig. 6). Two acetylated adducts were found in equal concentrations; one was identified as *N*-(deoxyguanosin-8-yl)-AAFBP while the other was proposed to be 3-(deoxyguanosin-N^2-yl)-AAFBP. At nontoxic doses (5 mg/kg), the nonacetylated adducts were lost from both tissues with a half-life of 10 days; *N*-(deoxyguanosin-8-yl)-AAFBP was removed much faster ($t_{1/2}$ = 2 days), while 3-(deoxyguanosin-N^2-yl)-AAFBP appeared to be a persistent lesion. When a 25 mg/kg dose was administered, there was an initial fast rate of removal of all adducts in the kidney which was attributed to a toxic response.

3,2′-Dimethyl-4-aminobiphenyl

3,2′-Dimethyl-4-aminobiphenyl (DMABP) is unique among arylamines in that it is primarily a colon carcinogen in rats (*33–35*). In a manner analogous to arylamine bladder carcinogens, DMABP has been proposed (*36*) to be *N*-oxidized and *N*-glucuronidated in the liver to yield *N*-hydroxy-DMABP-*N*-glucuronide, which is transported in the bile to the intestine where it can be

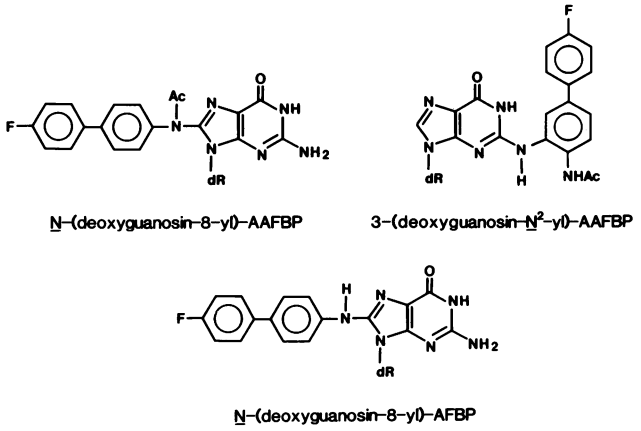

FIGURE 6. DNA adducts derived from 4-acetylamino-4′-fluorobiphenyl.

cleaved to release *N*-hydroxy-DMABP. Nussbaum et al. (*36*) have provided support for this mechanism by detecting *N*-hydroxy-DMABP-*N*-glucuronide as a biliary metabolite of DMABP. Flammang et al. (*37*) have shown that *N*-hydroxy-DMABP will react with DNA *in vitro* and that the extent of binding is greatly increased by AcCoA-dependent transacetylation that presumably yields *N*-acetoxy-DMABP. Further evidence for *N*-hydroxy-DMABP as a proximate carcinogen has been obtained by comparing hepatic and intestinal DNA adducts following a single subcutaneous injection of DMABP or its hydroxamic acid, *N*-acetyl-*N*-hydroxy-DMABP (*38*). In both tissues, two adducts were found: *N*-(deoxyguanosin-8-yl)-DMABP (80%) and 5-(deoxyguanosin-N^2-yl)-DMABP (20%) (Fig. 7). *N*-Acetylated adducts were not detected, and the extent of binding as well as the adduct profile obtained from *N*-acetyl-*N*-hydroxy-DMABP was nearly the same as that found with DMABP. These data suggest that *N*-hydroxy-DMABP serves as a common intermediate for both compounds.

The initial binding of DMABP to hepatic DNA was approximately twice that observed in intestinal DNA and the adducts in the liver were more persistent with only 30% being lost within 7 days as compared to 70% in the intestine. The greater persistence and higher adduct levels found in the liver appeared to be inconsistent with the tissue specificity of DMABP. However, when the rates of *de novo* DNA synthesis were measured, the rate in the intestine was at least 20 times that found in the liver. It seems, therefore, that although total binding is lower in the intestinal DNA, the highly

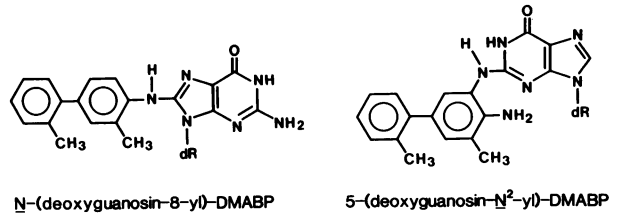

FIGURE 7. DNA adducts derived from 3,2′-dimethyl-4-aminobiphenyl.

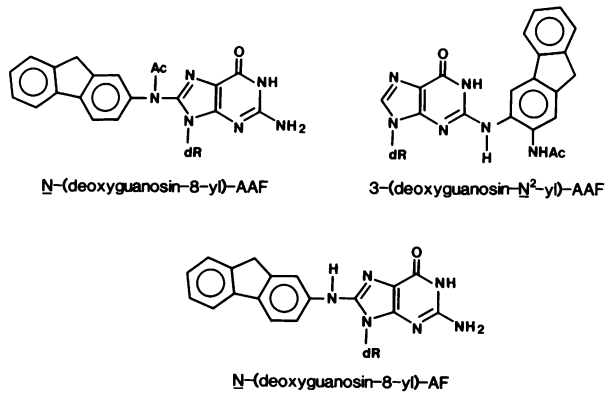

FIGURE 8. DNA adducts derived from 2-acetylaminofluorene.

proliferative nature of this tissue increases the probability that these adducts will serve as initiating lesions.

2-Acetylaminofluorene

2-Acetylaminofluorene (AAF), which is undoubtedly the most extensively studied of the arylamine carcinogens, induces tumors in a number of species and at a number of sites including liver, mammary gland, intestine, and urinary bladder (*39–41*). In rat liver, both acetylated and nonacetylated adducts are formed with the latter products accounting for approximately 80% of the total binding after a single administration of AAF or *N*-hydroxy-AAF (*42–44*). The major acetylated adduct in rat liver, which has been identified as *N*-(deoxyguanosin-8-yl)-AAF (Fig. 8), contributes about 15% of the total binding and has a half-life of 7 days *in vivo* (*43–45*). In contrast, the minor acetylated adduct, 3-(deoxyguanosin-N^2-yl)-AAF, which accounts for 5% of the binding, is a persistent lesion in hepatic DNA (*44,45*).

The majority of adducts found in rat liver are nonacetylated products that for many years resisted identification. Although they were suspected to be C8-deoxyguanosine derivatives, these adducts tended to undergo decomposition during DNA isolation and hydrolysis. It is now known that this was due to a base-catalyzed opening of the imidazole ring that yielded two pyrimidine adducts (*46*). When the isolation and hydrolysis were performed under milder conditions, chromatographic analysis of rat liver DNA indicated the presence of one major nonacetylated adduct, *N*-(deoxyguanosin-8-yl)-2-aminofluorene (AF) (*47,48*) (Fig. 8). This has been confirmed by both immunoassays (*50*) and ^{32}P-postlabeling (*51*) techniques. As was observed with 3-(deoxyguanosin-N^2-yl)-AAF, this nonacetylated adduct is relatively persistent in rat liver DNA (*47,49–52*).

Recently Gupta and Dighe (*24*) have examined in greater detail adduct formation and removal in rat liver DNA after a single intraperitoneal injection of *N*-hydroxy-AAF. While their data generally agree with earlier studies, there are a couple of important exceptions. First, due to the sensitivity of their ^{32}P-postlabeling method, they were able to detect many more adducts. Two of these, which accounted for 50% of the initial binding, were originally thought to be a new type of adduct but have subsequently been found to result from incomplete hydrolysis of the modified DNA (Gupta, personal communication). Nevertheless, it is clear from these results, as well as their earlier study (*51*), that a greater variety of adducts than originally suspected can be formed from AAF. Second, when adduct formation was measured as early as 30 min after treatment, there was a substantially greater proportion of acetylated adducts than previously reported.

In principle, a number of metabolic pathways can lead to the adducts detected in rat liver. Through the use of a sulfotransferase inhibitor, pentachlorophenol, Meerman et al. (*48,53*) have provided evidence that the predominant route to acetylated adducts is through the formation of *N*-sulfonyloxy-AAF. The situation regarding the nonacetylated adducts is less clear and evidence has been presented supporting *N*, *O*-acyltransferase-catalyzed formation of *N*-acetoxy-AF (*21,54,55*), direct reaction by *N*-hydroxy-AF (*56*), and AcCoA-dependent formation of *N*-acetoxy-AF(*22*).

Relatively few studies have been performed where hepatic adducts have been examined after multiple or chronic administration of AAF or *N*-hydroxy-AAF. In one experiment, both male and female Sprague-Dawley rats were administered up to four doses of *N*-hydroxy-AAF at biweekly intervals (*49*). *N*-(Deoxyguanosin-8-yl)-AAF was detected in the hepatic DNA from male

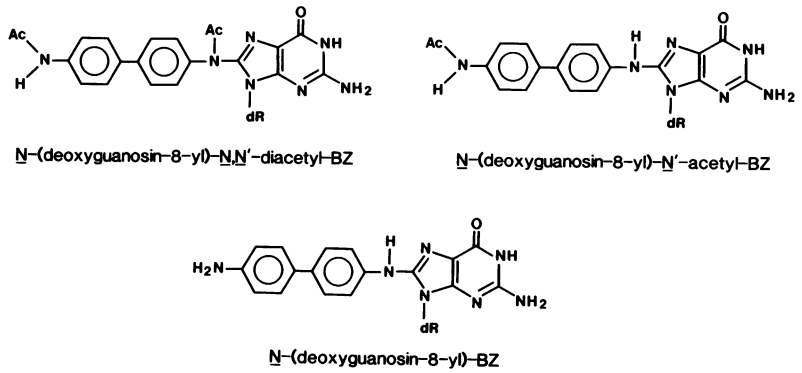

FIGURE 9. DNA adducts derived from benzidine.

rats only and only on the day after dosing, whereas 3-(deoxyguanosin-N^2-yl)-AAF could be found both 1 and 14 days after treatment and increased in an additive manner upon repeated administration of the carcinogen. *N*-(Deoxyguanosin-8-yl)-AF was the major adduct observed in the liver DNA from males and also appeared to increase in an additive manner. Only nonacetylated adducts were detected in the hepatic DNA from females, which is consistent with the low sulfotransferase levels in females of this strain (*57*). The total concentration of adducts was higher in females and these adducts tended to persist. Since females are resistant to *N*-hydroxy-AAF-induced hepatocarcinogenesis (*58*), these results emphasize that adduct persistence alone is not predictive of carcinogenesis.

In a series of experiments, Poirier et al. (*50,59,60*) have used immunoassays to analyze adduct formation and removal during dietary administration of AAF. When rats were given either 0.02 or 0.04% AAF, the concentration of adducts in the liver reached a plateau of about 300 pmole/mg DNA after approximately 3 weeks of feeding. Although acetylated adducts originally made a significant contribution to the total adduct concentration, by the time equilibrium was reached almost all of the adducts were nonacetylated. By using a combination of radioactive and nonradioactive AAF (*60*), they demonstrated that the capability of the liver to form and remove adducts remained constant for at least 1 month and that adduct removal did not depend upon continued carcinogen ingestion. In addition, they found the removal of adducts to be biphasic, which resulted in a significant proportion of the adducts being persistent. From these data a model was proposed in which there were two distinct regions in the genome: one susceptible to fast repair, and another where repair occurred much slower. Although a much greater number of adducts were formed in the fast repair region, the model predicted that the persistent lesions resided exclusively in the slow repair region.

The adducts formed from AAF or *N*-hydroxy-AAF have also been examined in rat kidney and mammary gland. After multiple doses of *N*-hydroxy-AAF or after dietary AAF, binding to kidney DNA was between 20 and 50 % of that found in hepatic DNA (*49,50*). The only adduct detected was *N*-(deoxyguanosin-8-yl)AF and this lesion persisted for at least 1 month following carcinogen administration. *N*-(Deoxyguanosin-8-yl)-AF was also the only adduct found in rat mammary gland epithelium after a single intraperitoneal dose of *N*-hydroxy-AAF (*61*). In this experiment, the maximum adduct concentration was found 4 hr after treatment and then decreased following first-order kinetics with a half-life of 14 days.

Among the aromatic amine carcinogens, AAF is unique in that it induces both bladder and liver tumors in dogs (*41*). In both tissues, *N*-(deoxyguanosin-8-yl)-AF was the major adduct detected after a single dose of AAF (*11*); it was the only adduct in epithelial DNA, whereas other minor adducts were found in hepatic DNA. When the adduct levels were compared at 2 and 7 days following administration, there was an 80% decrease in both tissues which could be due to DNA repair or cytotoxic responses. Since dogs readily deacetylate AAF (*26,27*), the formation of a nonacetylated adduct is consistent with *N*-hydroxy-AF being the reactive intermediate. However, it has recently been reported that dog bladders contain high levels of PHS (*18*) that can activate AF to DNA binding derivatives (*16*). The major adduct formed *in vitro* from PHS-catalyzed cooxidation appears to differ from *N*-(deoxyguanosin-8-yl)-AF (*62*), but its identity is not known. Whether or not this adduct is formed *in vivo* also remains to be established.

When *N*-hydroxy-AAF was administered intraperitoneally to 12-day-old $B6C3F_1$ mice, a high incidence of hepatic tumors resulted and this could be decreased by prior treatment with the sulfotransferase inhibitor, pentachlorophenol (*63*). In addition, only a low tumor incidence was found when *N*-hydroxy-AAF was administered to a sulfotransferase-deficient strain. These results suggest that *N*-sulfonyloxy-AAF should be the ultimate carcinogenic metabolite; however, when the hepatic adducts were examined, the major product detected was the nonacetylated derivative *N*-(deoxyguanosin-8-yl)-AF, with the acetylated adducts accounting for only 5% of the binding. Since pentachlorophenol decreased both the tumor yield and adduct formation, it appears that in these mice *N*-hydroxy-AAF is deacetylated to *N*-hydroxy-AF that is then converted to the ultimate carcinogen *N*-sulfonyloxy-AF. Support for this interpretation was provided by the observation that *N*-(deoxyguanosin-8-yl)-AF was formed in *in vitro* sulfotransferase assays conducted with mouse liver cytosol, *N*-hydroxy-AF and deoxyguanosine (*63*). Interestingly, when the model ultimate carcinogen *N*-acetoxy-AAF was administered to mice through the orbital sinus, the major adduct detected in lung (target) and liver (nontarget) DNA was *N*-(deoxyguanosin-8-yl)-AF, instead of the acetylated adducts which would have been obtained if this compound was reacted with DNA *in vitro* (Drinkwater and Beland, unpublished observations). The mechanism for this is not known but may involve *N*-deacetylation of *N*-acetoxy-AAF to *N*-acetoxy-AF.

Benzidine

Benzidine (BZ) is a urinary bladder carcinogen in humans (*64–66*) and dogs (*25*), and a hepatocarcinogen in rats, mice, and hamsters (*66–69*). Since BZ has two amino functions, its metabolic activation pathways are potentially more complicated than the monofunctional aromatic amines. In order to elucidate these pathways, BZ and its *N*-acetylated derivatives were administered to rats and the binding to hepatic DNA was compared (*70–72*). The relative order of binding was *N*-acetyl-BZ> BZ >> *N*, *N'*-diacetyl-BZ, which indicated that monoacetylation was part of the activation sequence but that the addition of a second acetyl group resulted in a detoxified product. Based upon this information, *N*-hy-

droxy-N'-acetyl-BZ, a suspected activated derivative of N-acetyl-BZ was synthesized, reacted with DNA, and the adduct profile was compared to that obtained *in vivo*. Using this approach, the major *in vivo* adduct was identified as N-(deoxyguanosin-8-yl)-N'-acetyl-BZ (Fig. 9), a product derived either directly from N-hydroxy-N'-acetyl-BZ or after O-esterification of this N-hydroxy arylamine. When the extent of binding was compared at 1 and 7 days after a single intraperitoneal injection of N-acetyl-BZ, the concentration of this adduct was found to decrease by 60%. Similar kinetics of removal had been observed earlier by Martin and Ekers (*73*), who found a 40% decrease in hepatic DNA binding between 1 and 2 days and then a constant binding level for at least an additional 4 weeks. Subsequently, by using N, N'-diacetylbenzidine with higher specific activity, a second adduct, N-(deoxyguanosin-8-yl)-N,N'-diacetyl-BZ, was detected in rat liver *in vivo* at approximately 15% of the level of the major adduct (*72*).

The adducts formed from BZ or its N-acetylated derivatives have also been examined in other experimental animals. In mice, a single adduct, N-(deoxyguanosin-8-yl)-N'-acetyl-BZ, was found following administration of BZ in the drinking water for 1 week (*70*). The highest binding was observed immediately after treatment; it decreased by about 50% in 1 day, and then remained at a constant level for at least an additional week. The extent of binding and kinetics of removal of this adduct were the same in male and female mice, although females were much more susceptible to hepatic tumor induction by BZ (*69*). In hamsters (*72*), the same adduct, N-(deoxyguanosin-8-yl)-N'-acetyl-BZ was found in liver DNA following a single IP injection of N-acetyl-BZ. The concentration of this adduct was 50% of that observed in rats, but the kinetics of removal appeared similar with approximately a 50% reduction occurring between 1 and 7 days after treatment.

BZ-derived adducts have also been examined in dogs (*11*). As noted earlier, BZ is a bladder carcinogen in this species (*25*) which may be due to their low N-acetylase activity (*26,27*). When a single dose of BZ was given to dogs, a low level of binding could be detected in urothelial DNA (*11*). Although a much higher binding was observed when N-acetyl-BZ was given, $< 10\%$ appeared to be N-(deoxyguanosin-8-yl)-N'-acetyl-BZ. Both BZ and N-acetyl-BZ gave very high levels of hepatic DNA binding but $< 1\%$ could be released upon a standard enzymatic hydrolysis as opposed to the 70 to 90% with other arylamine carcinogens. The identity of these products has not yet been determined.

Since high levels of PHS have been detected in dog bladder (*18*), cooxidation of BZ by this enzyme could result in some of the binding observed *in vivo*. In addition, the liver has a variety of peroxidases that could catalyze the hepatic oxidation of BZ to DNA-binding derivatives. Peroxidase-mediated cooxidation of BZ *in vitro* has been demonstrated (*18,74*), and a major DNA adduct arising from this oxidation has recently been identified as N-(deoxyguanosin-8-yl)-BZ (Fig. 9) (*75*). Whether or not this adduct accounts for some of the unidentified adducts in the bladder and liver remains to be determined. The presence of two amine functions in BZ also offers the possibility that dyelike crosslinked products (i.e., DNA–DNA or DNA–protein) may be formed during its oxidation. This would presumably be a minor pathway in rodent species that readily N-acetylate BZ.

N,N-Dimethyl-4-aminoazobenzene, *N*-Methyl-4-aminoazobenzene and 4-Aminoazobenzene

N,N-Dimethyl-4-aminoazobenzene (DAB) was among the first carcinogens found that induced tumors at a location removed from the site of application (*76*). This observation, plus DAB's high molar extinction coefficient at visible wavelengths (which facilitated its study in the days preceding radiolabeled compounds), resulted in a number of investigations being conducted with this aminoazo dye (*2*). The first step in the metabolic activation of DAB is a cytochrome P-450-catalyzed oxidative N-demethylation to N-methyl-4-aminoazobenzene (MAB) (*2,77*). Since a second N-demethylation, which yields 4-aminoazobenzene (AB), generally results in a decrease in carcinogenicity, most studies have concentrated on MAB, which is further activated by FMO-catalyzed N-oxidation to N-hydroxy-MAB and then is converted to an ultimate carcinogen through PAPS-dependent formation of N-sulfonyloxy-MAB.

A number of adducts have been characterized from MAB *in vivo*. In rat liver, a target for MAB, two adducts were detected following the administration of a single oral dose. The major adduct, N-(deoxyguanosin-8-yl)-MAB (Fig. 10), which was first described by Lin et al. (*78*), initially accounted for 70% of the bound material but was rapidly removed and could not be detected 7 days after treatment (*77–79*). The second adduct was identified as 3-(deoxyguanosin-N^2-yl)-MAB and was found to be a persistent lesion whose concentration remained constant for at least 2 weeks (*77,79*). When multiple doses of MAB were administered and the adducts examined 8 hr after treatment, a third product was detected and subsequently identified as 3-(deoxyadenosin-N^6-yl)-MAB (*80*). Each of the adducts increased in concentration with repeated administration with the rate of increase being faster with 3-(deoxyguanosin-N^2-yl)-MAB and 3-(deoxyadenosin-N^6-yl)-MAB. These kinetics are consistent with the rapid repair of N-(deoxyguanosin-8-yl)-MAB in rat liver. More recently, the adduct profile has been determined after feeding MAB to Sprague-Dawley rats for 1,3, or 5 weeks (*81*). Immediately after treatment, the binding to hepatic DNA was 3- to 10-fold higher than that found in the nontarget tissues, kidney and spleen. Within 3 days the hepatic adduct concentration had decreased by 50%; and when the adduct profiles were determined, the only adduct that appeared to persist was 3-(deoxyguanosin-N^2-yl)-MAB.

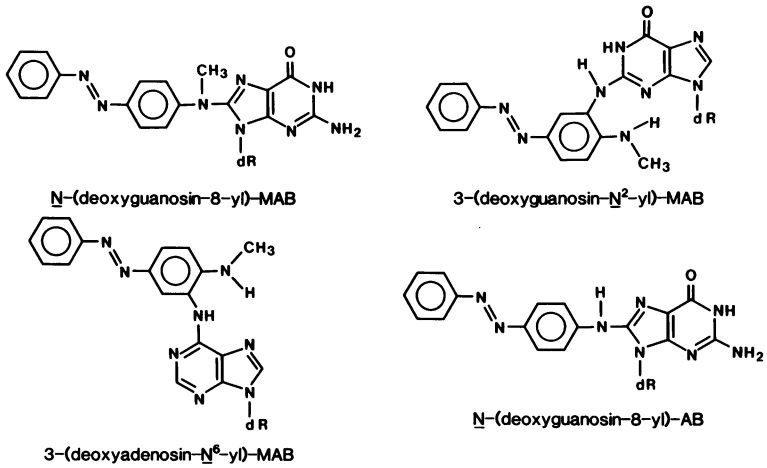

FIGURE 10. DNA adducts derived from *N,N*-dimethyl-4-aminoazobenzene, *N*-methyl-4-aminoazobenzene and 4-aminoazobenzene.

Tarpley et al. (*82*) have examined the hepatic DNA adducts in 12-day-old mice administered a single IP injection of either MAB or DAB. At least 12 products were detected due, in part, to a *cis-trans* isomerism about the azo linkage. As had been observed with rats, the major adduct was *N*-(deoxyguanosin-8-yl)-MAB, which was rapidly removed from the DNA with 70% being lost within 10 days after administration. Significant quantities of 3-(deoxyguanosin-N^2-yl)-MAB were also found, and in a manner similar to what was found in rat liver, this adduct was relatively persistent with only 30% being removed within 10 days.

Although AB is not carcinogenic to adult rats, recent studies indicate that it is nearly as hepatocarcinogenic as MAB and DAB in preweanling mice (*83,84*). When the hepatic DNA adducts were examined 1 day following a single IP injection of AB, only one adduct, *N*-(deoxyguanosin-8-yl)-AB, was detected (*84*). The same adduct was found in DAB-treated mice, although it was only about 25% of the level observed in mice administered AB. In addition, two other adducts were identified in the DAB-treated animals: *N*-(deoxyguanosin-8-yl)-MAB and 3-(deoxyguanosin-N^2-yl)-MAB. In light of the observation that *N*-hydroxy-AF is activated by formation of a sulfate ester in mouse liver and that this tissue contains very low levels of *N,O*-acyltransferase and acetyl CoA-dependent *O*-acetylase, it appears likely that the major pathway leading to hepatic AB adducts is through the formation of *N*-sulfonyloxy-AB.

2-Acetylaminophenanthrene

2-Acetylaminophenanthrene(AAP) is another aromatic amide which gives substantial levels of hepatic DNA binding (*24,85,86*) although it is not hepatocarcinogenic, either with (*86,87*) or without (*88*) promotion. In order to determine the reason for this, Scribner and Koponen (*85*) compared the hepatic DNA adducts formed from AAP to those obtained from AAF following a single IP injection to male Fischer rats. Both compounds gave approximately equal levels of binding and a substantial amount of persistent adducts; however, only nonacetylated adducts, of which two were major and two were minor, were detected from AAP. Equal levels of binding were also detected when rats were fed either of the aromatic amides (*86*). More recently, Gupta and Dighe have reexamined the kinetics of formation and removal of AAP adducts following a single administration of *N*-hydroxy-AAP (*24*). At 4 hr, the hepatic DNA binding of *N*-hydroxy-AAP was much lower (approximately 10%) than that observed with *N*-hydroxy-AAF. However, by 24 hr the binding was nearly equal, and, 10 days after carcinogen administration, the binding detected from *N*-hydroxy-AAP was substantially higher than that found with *N*-hydroxy-AAF. Examination of the adduct profile revealed two major and at least eight minor adducts all of which could be formed by reacting *N*-hydroxy-2-aminophenanthrene (AP) with calf thymus DNA. Thus, in accord with the earlier results of Scribner and Koponen (*85*), all of the adducts appeared to be nonacetylated. Further insight into the identity of the adducts was obtained by reacting *N*-hydroxy-AP with synthetic deoxynucleoside polymers which indicated that the two major adducts were derived from substitution of deoxyguanosine. Preliminary spectroscopic data suggest that one of these products is *N*-(deoxyguanosin-8-yl)-AP (*89*) (Fig. 11), similar to a guanosine product originally reported by Scribner et al. (*86*).

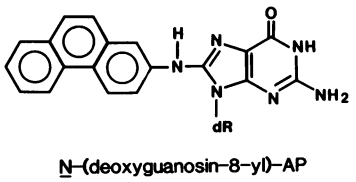

FIGURE 11. DNA adduct derived from 2-acetylaminophenanthrene.

Conclusions

Over the last decade, the identification of aromatic amine-DNA adducts has provided new insights into the mechanisms of metabolic activation, mutagenicity and carcinogenicity. In general, the major sites of carcinogen modification in DNA have been C8 and N^2 of guanine, with substitution at O^6 of guanine, and N^6 and C8 of adenine occurring to a lesser extent. Depending upon the metabolic activation pathway involved, the aromatic amino groups of these adducts have been found to be acetylated, nonacetylated or methylated. In fact, the identity of these adducts, along with the overall adduct profiles observed *in vivo*, has provided strong evidence for the role of specific metabolic conjugation reactions that have been demonstrated *in vitro*.

Although target-tissue specificity of aromatic amine carcinogens has correlated with the levels of DNA modification and with the relative persistence of many of these adducts, several exceptions have been noted in this review. Thus, it is clear that adduct formation *per se* is not sufficient for tumor initiation (fixation of a heritable lesion that can lead to neoplasia) and that other processes, such as error-prone repair and cellular replication, play an essential role. In addition, the relative biological potency of aromatic amine–DNA adducts may differ appreciably from one another as evidenced by the differing mutation efficiencies in *Salmonella typhimurium* TA1538 and TA1535 (*11*), and in Chinese hamster ovary cells (*90*). Furthermore, mutational hotspots have been found in aromatic amine-modified DNA (*91*), which indicates that the biological activity of adducts is further influenced by specific DNA sequences.

Analysis of carcinogen-induced changes in DNA sequence (*91–93*) has often revealed base deletions (frameshifts) which is consistent with *in vitro* studies in which the replication of aromatic amine-modified DNA has resulted in chain termination (*94,95*). In addition, aromatic amine substitution has also led to transversion mutations (*93*). These aromatic amine-induced mutations are thought to arise from conformational changes in DNA, including conversion of the modified base from an *anti* to *syn* conformation (*11,91,94,96*). This, in turn, results in alterations in DNA conformation (B to Z; B/Z junctions; altered B-form) which has been suggested to lead to base deletions and specific mispairings (*94,97–100*). For example, C8 and N^2-substituted guanines have been predicted to result in G to T or G to C transversions (*93,99*), O^6-modified guanines in G to A transitions (*8*), and N^6- and C8-substituted adenines in A to T or A to C transversions (*99*). Therefore, it may not be coincidental that activation of cellular transforming genes of the *ras* family (c-ras^H, c-ras^K, and c-ras^N), which has been implicated in bladder, colon, liver, mammary, lung, nervous system and embryonal tumors, can be achieved by G to T transversions (*101–103*) and G to A transitions (*104,105*) at coding position 12 and A to T transversions (*106*) at coding position 61 of the first exon. Additional studies of the consequences of aromatic amine–DNA adducts should be pursued and may provide important data on the molecular biology of cancer.

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REFERENCES

1. Radomski, J. R. The primary aromatic amines: their biological properties and structure-activity relationships. Ann. Rev. Pharmacol. Toxicol. 19: 129–157 (1979).
2. Miller, J. A., and Miller, E. C. Some historical aspects of *N*-aryl carcinogens and their metabolic activation. Environ. Health Perspect. 49: 3–12 (1983).
3. Radomski, J. L., Deichmann, W. B., Altman, N. H., and Radomski, T. Failure of pure 1-naphthylamine to induce bladder tumors in dogs. Cancer Res. 40: 3537–3539 (1980).
4. Purchase, I. F. H., Kalinowski, A. E., Ishmael, J., Wilson, J., Gore, C. W., and Chart, I. S. Lifetime carcinogenicity study of 1- and 2-naphthylamine in dogs. Brit. J. Cancer 44: 892–901 (1981).
5. Belman, S., Troll, W., Teebor, G., and Mukai, F. The carcinogenic and mutagenic properties of *N*-hydroxy-aminonaphthalenes. Cancer. Res. 28: 535–542 (1968).
6. Radomski, J. L., Brill, E., Deichmann, W. B., and Glass, E. M. Carcinogenicity testing of *N*-hydroxy and other oxidation and decomposition products of 1- and 2-naphthylamine. Cancer Res. 31: 1461–1467 (1971).
7. Dooley, K. L., Beland, F. A., Bucci, T. J., and Kadlubar, F. F. Local carcinogenicity, rates of absorption, extent and persistence of macromolecular binding, and acute histopathological effects of *N*-hydroxy-1-naphthylamine and *N*-hydroxy-2-naphthylamine. Cancer Res. 44: 1172–1177 (1984).
8. Kadlubar, F. F., Miller, J. A., and Miller, E. C. Guanyl O^6-arylamination and O^6-arylation of DNA by the carcinogen *N*-hydroxy-1-naphthylamine. Cancer Res. 38: 3628–3638 (1978).
9. Murofushi, Y., Hashimoto, Y., Shudo, K., and Okamoto, T. Reaction of 1-naphthylhydroxyamine with calf thymus deoxyribonucleic acid. Isolation and synthesis of N-(guanin-C^8-yl)-1-naphthylamine. Chem. Pharm Bull. 29: 2730–2732 (1981).
10. Kadlubar, F. F., Anson, J. F., Dooley, K. L., and Beland, F. A. Formation of urothelial and hepatic DNA adducts from the carcinogen 2-naphthylamine. Carcinogenesis 2: 467–470 (1981).
11. Beland, F. A., Beranek, D. T., Dooley, K. L., Heflich, R. H., and Kadlubar, F. F. Arylamine-DNA adducts *in vitro* and *in vivo*: their role in bacterial mutagenesis and urinary bladder carcinogenesis. Envir. Health Perspect. 49: 125–134 (1983).
12. Hammons, G. J., Guengerich, F. P., Weis, C. C., Beland, F. A., and Kadlubar, F. F. Mutagenic oxidation of carcinogenic arylamines by rat, dog, and human hepatic microsomes and by purified flavin-containing and cytochrome P-450 monooxygenases. Cancer Res. 45: 3578–3585 (1985).
13. Kadlubar, F. F., Unruh, L. E., Beland, F. A., Straub, K. M., and Evans, F. E. *In vitro* reaction of the carcinogen, *N*-hydroxy-2-naphthylamine, with DNA at the C-8 and N^2 atoms of guanine and at the N^6 atom of adenine. Carcinogenesis 1: 139–150 (1980).
14. Kadlubar, F. F., Miller, J. A., and Miller, E. C. Hepatic microsomal *N*-glucuronidation and nucleic acid binding of *N*-hydroxy arylamines in relation to urinary bladder carcinogenesis. Cancer. Res. 37: 805–814 (1977).
15. Young, J. F., and Kadlubar, F. F. A pharmacokinetic model to predict exposure of the bladder epithelium to urinary N-hydroxyarylamine carcinogens as a function of urine pH, voiding interval, and resorption. Drug. Metab. Disposit. 10: 641–644 (1982).
16. Kadlubar, F. F., Frederick, C. B., Weis, C. C., and Zenser, T. V. Prostaglandin endoperoxide synthetase-mediated metabolism of carcinogenic aromatic amines and their binding to DNA and protein. Biochem. Biophys. Res. Commun. 108: 253–258 (1982).
17. Yamazoe, Y., Miller, D. W., Weis, C. C., Dooley, K. L., Zenser, T. V., Beland, F. A., and Kadlubar, F. F. DNA adducts formed by ring-oxidation of the carcinogen 2-naphthylamine with prostaglandin-H synthase *in vitro* and in the dog urothelium *in vivo*, Carcinogenesis, in press.
18. Wise, R. W., Zenser, T. V., Kadlubar, F. F., and Davis, B. B. Metabolic activation of carcinogenic aromatic amines by dog bladder and kidney prostaglandin H synthase. Cancer Res. 44: 1893–1897 (1984).

19. Shirai, T., Lee, M.-S., Wang, C. Y., and King, C. M. Effects of partial hepatectomy and dietary phenobarbital on liver and mammary tumorigenesis by two *N*-hydroxy-*N*-acylaminobiphenyls in female CD rats. Cancer Res. 41: 2450–2456 (1981).
20. Kriek, E. On the mechanism of action of carcinogenic aromatic amines. II. Binding of *N*-hydroxy-*N*-acetyl-4-aminobiphenyl to rat-liver nucleic acids *in vivo*. Chem.-Biol. Interact. 3: 19–28 (1971).
21. King, C. M., and Glowinski, I. B. Acetylation, deacetylation and acyltransfer. Environ. Health Perspect. 43: 42–50 (1983).
22. Flammang, T. J., and Kadlubar, F. F. Acetyl Co A-dependent, cytosol-catalyzed binding of carcinogenic N-hydroxy-arylamines to DNA. In: Microsomes and Drug Oxidations (A. R. Boobis, J. Caldwell, F. DeMatteis, and C. R. Elcombe, Eds.), Taylor and Francis, London, 1985, pp. 190–197.
23. Kriek, E., and Westra, J. G. Metabolic activation of aromatic amines and amides and interactions with nucleic acids. In: Chemical Carcinogens and DNA, Vol. II (P. L. Grover, Ed.), CRC Press, Boca Raton, FL, 1979, pp. 1–28.
24. Gupta, R. C., and Dighe, N. R. Formation and removal of DNA adducts in rat liver treated with N-hydroxy derivatives of 2-acetylaminofluorene, 4-acetylaminobiphenyl, and 2-acetylaminophenanthrene. Carcinogenesis 5: 343–349 (1984).
25. Price, J. M. Etiology of bladder cancer. In: Benign and Malignant Tumors of the Urinary Bladder (E. Maltry, Jr., Ed.), Medical Examination Publ. Co., Flushing, NY, 1971, pp. 189–261.
26. Poirier, L. A., Miller, J. A., and Miller, E. C. The *N*- and ring-hydroxylation of 2-acetylaminofluorene and the failure to detect *N*-acetylation of 2-aminofluorene in the dog. Cancer Res. 23: 790–800 (1963).
27. Lower, G. M., Jr., and Bryan, G. T. Enzymatic *N*-acetylation of carcinogenic aromatic amines by liver cytosol of species displaying different organ susceptibilities. Biochem. Pharmacol. 22: 1581–1588 (1973).
28. Kadlubar, F. F., Beland, F. A., Beranek, D. T., Dooley, K. L., Heflich, R. H., and Evans, F. E. Arylamine-DNA adduct formation in relation to urinary bladder carcinogenesis and *Salmonella typhimurium* mutagenesis. In: Environmental Mutagens and Carcinogens, (T. Sugimura, S. Kondo, and H. Takebe, Eds.), Alan R. Liss, New York, 1982, pp. 385–396.
29. Stromberg, K., and Reuber, M. D. Influence of age and sex on hepatic lesions induced by chemical carcinogens: injestion of *N*-4-(4′-fluorobiphenyl)acetamide by Buffalo strain rats. J. Natl. Cancer Inst. 44: 1047–1054 (1970).
30. Reuber, M. D. Hyperplastic and neoplastic lesions of the kidney in Buffalo rats in varying ages ingesting *N*-4-(4′-fluorobiphenyl)acetamide. J. Natl. Cancer Inst. 54: 427–429 (1975).
31. Stromberg, K., and Reuber, M. D. Histopathology of breast lesions induced in BUF rats of varying ages by ingestion of *N*-4-(4′-fluorobiphenyl)acetamide. J. Natl. Cancer Inst. 54: 1223–1230 (1975).
32. Kriek, E., and Hengeveld, G. M. Reaction products of the carcinogen *N*-hydroxy-4-acetylamino-4′-fluorobiphenyl with DNA in liver and kidney of the rat. Chem.-Biol. Interact. 21: 179–201 (1978).
33. Walpole, A. L., Williams, M. H. C., and Roberts, D. C. The carcinogenic action of 4-aminodiphenyl and 3:2′dimethyl-4-aminodiphenyl. Brit. J. Ind. Med. 9: 255–263 (1952).
34. Walpole, A. L., Williams, M. H. C., Roberts, D. C., and Hendry, J. A. Experiments in carcinogenesis with methyl derivatives of 4-aminodiphenyl. Acta Un. Int. Cancer 10: 174–175 (1954).
35. Walpole, A. L., Williams, M. H. C., and Roberts, D. C. Bladder tumours induced in rats of two strains with 3:2′-dimethyl-4-aminodiphenyl. Brit. J. Cancer 9: 170–176 (1955).
36. Nussbaum, M., Fiala, E. S., Kulkarni, B., El-Bayoumy, K., and Weisburger, J. H. *In vivo* metabolism of 3,2′-dimethyl-4-aminobiphenyl (DMAB) bearing on its organotropism in the Syrian golden hamster and the F344 rat. Environ. Health Perspect. 49: 223–231 (1983).
37. Flammang, R. J., Westra, J. G., Kadlubar, F. F., and Beland, F. A. DNA adducts formed from the probable proximate carcinogen, *N*-hydroxy-3,2′-dimethyl-4-aminobiphenyl, by acid catalysis or S-acetyl coenzyme A-dependent enzymatic esterification. Carcinogenesis, 6: 251–258 (1985).
38. Westra, J. G., Flammang, T. J., Fullerton, N. F., Beland, F. A., Weis, C. C., and Kadlubar, F. F. Formation of DNA adducts *in vivo* in rat liver and intestinal epithelium after administration of the carcinogen 3,2′-dimethyl-4-aminobiphenyl and its hydroxamic acid. Carcinogenesis, 6: 37–44 (1985).
39. Wilson, R. H., DeEds, F., and Cox, A. J. Jr. The toxicity and carcinogenic activity of 2-acetaminofluorene. Cancer Res. 1: 595–608 (1941).
40. Bonser, G. M., and Green, H. N. Epithelial neoplastic changes in the urinary tract of rabbits induced by feeding with 2-acetylaminofluorene. J. Pathol. Bacteriol. 62: 531–539 (1950).
41. Morris, H. P., and Eyestone, W. H. Tumors of the liver and urinary bladder of the dog after ingestion of 2-acetylaminofluorene. J. Natl. Cancer Inst. 13: 1139–1165 (1953).
42. Irving, C. C., and Veazey, R. A. Persistent binding of 2-acetylaminofluorene to rat liver *in vivo* and consideration of the mechanism of binding of *N*-hydroxy-2-acetylaminofluorene to rat liver nucleic acids. Cancer Res. 29: 1799–1804 (1969).
43. Kriek, E. On the mechanism of action of carcinogenic aromatic amines. I. Binding of 2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene to rat-liver nucleic acids *in vivo*. Chem.-Biol. Interact. 1: 3–17 (1969).
44. Kriek, E. Persistent binding of a new reaction product of the carcinogen *N*-hydroxy-*N*-2-acetylaminofluorene with guanine in rat liver DNA *in vivo*. Cancer Res. 32: 2042–2048 (1972).
45. Westra, J. G., Kriek, E., and Hittenhausen, H. Identification of the persistently bound form of the carcinogen N-acetyl-2-aminofluorene to rat liver DNA in vivo. Chem.-Biol. Interact. 15: 149–164 (1976).
46. Kriek, E., and Westra, J. G. Structural identification of the pyrimidine derivatives formed from N-(deoxyguanosin-8-yl)-2-aminofluorene in aqueous solution at alkaline pH. Carcinogenesis 1: 459–468 (1980).
47. Visser, A., and Westra, J. G. Partial persistency of 2-aminofluorene and *N*-acetyl-2-aminofluorene in rat liver DNA. Carcinogenesis 2: 737–740 (1981).
48. Meerman, J. H. N., Beland, F. A., and Mulder, G. J. Role of sulfation in the formation of DNA adducts from N-hydroxy-2-acetylaminofluorene in rat liver *in vivo*. Inhibition of N-acetylated aminofluorene adduct formation by pentachlorophenol. Carcinogenesis 2: 413–416 (1981).
49. Beland, F. A., Dooley, K. L., and Jackson, C. D. Persistence of DNA adducts in rat liver and kidney after multiple doses of the carcinogen N-hydroxy-2-acetylaminofluorene. Cancer Res. 42: 1348–1354 (1982).
50. Poirier, M. C., True, B. A., and Laishes, B. A. Formation and removal of (guan-8-yl)-DNA-2-acetylaminofluorene adducts in liver and kidney of male rats given dietary 2-acetylaminofluorene. Cancer Res. 42: 1317–1321 (1982).
51. Gupta, R. C., Reddy, M. V., and Randerath, K. ^{32}P-postlabeling of non-radioactive carcinogen-DNA adducts. Carcinogenesis 3: 1081–1092 (1982).
52. Beland, F. A., Dooley, K. L., and Casciano, D. A. Rapid isolation of carcinogen-bound DNA and RNA by hydroxyapatite chromatography. J. Chromtogr. 174: 177–186 (1979).
53. Meerman, J. H. N., van Doorn, A. B. D., and Mulder, G. J. Inhibition of sulfate conjugation of *N*-hydroxy-2-acetylaminofluorene in isolated perfused rat liver and in the rat *in vivo* by pentachlorophenol and low sulfate. Cancer Res. 40: 3772–3779 (1980).
54. King, C. M. Mechanism of reaction, tissue distribution, and inhibition of arylhydroxamic acid acyltransferase. Cancer Res. 34: 1503–1515 (1974).
55. Allaben, W. T., and King, C. M. The purification of rat liver arylhydroxamic acid *N*, *O*-acyltransferase. J. Biol Chem. 259: 12128–12134 (1984).
56. Frederick, C. B., Mays, J. B., Ziegler, D. M., Guengerich, F. P., Kadlubar, F. F. Cytochrome P-450- and flavin-containing monooxygenase-catalyzed formation of the carcinogen *N*-hydroxy-2-aminofluorene and its covalent binding to nuclear DNA. Cancer Res. 42: 2671–2677 (1982).
57. DeBaun, J. R., Miller, E. C., and Miller, J. A. *N*-Hydroxy-2-

acetylaminofluorene sulfotransferase: its probable role in carcinogenesis and protein-(methion-S-yl) binding in rat liver. Cancer Res. 30: 577–595 (1970).
58. Miller, E. C., Miller, J. A., and Hartman, H. A. *N*-Hydroxy-2-acetylaminofluorene: a metabolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. Cancer Res. 21: 815–824 (1961).
59. Poirier, M. C., True, B. A., and Laishes, B. A. Determination of 2-acetylaminofluorene adducts by immunoassay. Environ. Health Perspect. 49: 93–99 (1983).
60. Poirier, M. C., Hunt, J. M., True, B. A., Laishes, B. A., Young, J. F., and Beland, F. A. DNA adduct formation, removal and persistence in rat liver during one month of feeding 2-acetylaminofluorene. Carcinogenesis 5: 1591–1596 (1984).
61. Allaben, W. T., Weis, C. C., Fullerton, N. F., and Beland, F. A. Formation and persistence of DNA adducts from the carcinogen N-hydroxy-2-acetylaminofluorene in rat mammary gland *in vivo*. Carcinogenesis 4: 1067–1070 (1983).
62. Krauss, R. S., and Eling, T. S. Formation of unique arylamine/DNA adducts from 2-aminofluorene activated by prostaglandin H synthase. Cancer Res. 45: 1680–1686 (1985).
63. Lai, C.-C., Miller, E. C., Miller, J. A., and Liem, A. Strong evidence that N-sulfoxy-2-aminofluorene is the major ultimate carcinogenic metabolite of N-hydroxy-2-acetylaminofluorene (N-HO-AAF) in infant male mice. Proc. Am. Assoc. Cancer Res. 25: 85 (1984).
64. Case, R. A. M., Hosker, M. E., MacDonald, D. B., and Pearson, J. T. Tumours of the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British chemical industry. Part I. The role of aniline, benzidine, alpha-naphthylamine and beta-naphthylamine. Brit. J. Ind. Med. 11: 75–104 (1954).
65. Mancuso, T. F., and El Attar, A. A. Cohort study of workers exposed to beta-naphthylamine and benzidine. J. Occup. Med. 9: 277–285 (1967).
66. Haley, T. J. Benzidine revisited: a review of the literature and problems associated with the use of benzidine and its congeners. Clin. Toxicol. 8: 13–42 (1975).
67. Spitz, S., Maguigan, W. H., and Dobriner, K. The carcinogenic action of benzidine. Cancer 3: 789–804 (1950).
68. Vesselinovitch, S. D., Rao, K. V. N., and Mihailovich, N. Factors modulating benzidine carcinogenicity bioassay. Cancer Res. 35: 2814–2819 (1975).
69. Nelson, C. J., Baetcke, K. P., Frith, C. H., Kodell, R. L., and Schieferstein, G. The influence of sex, dose, time, and cross on neoplasia in mice given benzidine dihydrochloride. Toxicol. Appl. Pharmacol. 64: 171–186 (1982).
70. Martin, C. N., Beland, F. A., Roth, R. W., and Kadlubar, F. F. Covalent binding of benzidine and *N*-acetylbenzidine to DNA at the C-8 atom of deoxyguanosine *in vivo* and *in vitro*. Cancer Res. 42: 2678–2686 (1982).
71. Martin, C. N., Beland, F. A., Kennelly, J. C., and Kadlubar, F. F. Binding of benzidine, *N*-acetylbenzidine, *N,N'*-diacetylbenzidine and Direct Blue 6 to rat liver DNA. Environ. Health Perspect. 49: 101–106 (1983).
72. Kennelly, J. C., Beland, F. A., Kadlubar, F. F., and Martin, C. N. Binding of N-acetylbenzidine and N,N'-diacetylbenzidine to hepatic DNA of rat and hamster in vivo and in vitro. Carcinogenesis 5: 407–412 (1984).
73. Martin, C. N., and Ekers, S. F. Studies on the macromolecular binding of benzidine. Carcinogenesis 1: 101–109 (1980).
74. Morton, K. C., King, C. M., Vaught, J. B., Wang, C. Y., Lee, M.-S., and Marnett, L. J. Prostaglandin H synthase-mediated reaction of carcinogenic arylamines with tRNA and homopolyribonucleotides. Biochem. Biophys. Res. Commun. 111: 96–103 (1983).
75. Yamazoe, Y., Beland, F. A. and Kadlubar, F. F. Evidence for benzidine diimine as a reactive intermediate in the peroxidase-mediated binding of benzidine to DNA. Proc. Am. Assoc. Cancer Res. 26: 85 (1985).
76. Kinosita, R. Studies on the cancerogenic chemical substances. Japan Pathol. Soc. Trans. 27: 665–725 (1937).
77. Beland, F. A., Tullis, D. L., Kadlubar, F. F., Straub, K. M., and Evans, F. E. Identification of the DNA adducts formed in vitro from *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene and in rat liver in vivo after administration of *N*-methyl-4-aminoazobenzene. J. Natl. Cancer. Inst. Monographs 58: 153–161 (1982).
78. Lin, L.-K., Miller, J. A., and Miller, E. C. Structures of hepatic nucleic acid-bound dyes in rats given the carcinogen *N*-methyl-4-aminoazobenzene. Cancer Res. 35: 844–850 (1975).
79. Beland, F. A., Tullis, D. L., Kadlubar, F. F., Straub, K. M., Evans, F. E. Characterization of DNA adducts of the carcinogen *N*-methyl-4-aminoazobenzene in vitro and in vivo. Chem.-Biol. Interact. 31: 1–17 (1980).
80. Tullis, D. L., Straub, K. M., and Kadlubar, F. F. A comparison of the carcinogen-DNA adducts formed in rat liver in vivo after administration of single or multiple doses of *N*-methyl-4-aminoazobenzene. Chem.-Biol. Interact. 38: 15–27 (1981).
81. Tullis, D. L., Baetcke, K. P., and Kadlubar, F. F. Formation and biological properties of DNA adducts from the hepatocarcinogen, N-methyl-4-aminoazabenzene. Proc. Am. Assoc. Cancer Res. 23: 62 (1982).
82. Tarpley, W. G., Miller, J. A., and Miller, E. C. Adducts from the reaction of *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene with deoxyguanosine or DNA *in vitro* and from hepatic DNA of mice treated with *N*-methyl- or *N,N*-dimethyl-4-aminoazobenzene. Cancer Res. 40: 2493–2499 (1980).
83. Miller, E. C., Kadlubar, F. F., Miller, J. A., Pitot, H. C., and Drinkwater, N. R. The N-hydroxy metabolites of N-methyl-4-aminoazobenzene and related dyes as proximate carcinogens in the rat and mouse. Cancer Res. 39: 3411–3418 (1979).
84. Delclos, K. B., Tarpley, W. G., Miller, E. C., and Miller, J. A. 4-Aminoazobenzene and *N,N*-dimethyl-4-aminoazobenzene as equipotent hepatic carcinogens in male C57BL/6 × C3H/He F_1 mice and characterization of *N*-(deoxyguanosin-8-yl)-4-aminoazobenzene as the major persistent hepatic DNA-bound dye in these mice. Cancer Res. 44: 2540–2550 (1984).
85. Scribner, J. D., and Koponen, G. Binding of the carcinogen 2-acetamidophenanthrene to rat liver nucleic acids: lack of correlation with carcinogenic activity, and failure of the hydroxamic acid ester model for in vivo activation. Chem.-Biol. Interact. 28: 201–209 (1979).
86. Scribner, J. D., Woodworth, B., Koponen, G., and Holmes, E. H. Use of 2-acetamidophenanthrene and 2-acetamidofluorene in investigations of mechanisms of hepatocarcinogenesis. Environ. Health Perspect. 49: 81–86 (1983).
87. Scribner, J. D., and Mottet, N. K. DDT acceleration of mammary gland tumors induced in the male Sprague-Dawley rat by 2-acetamidophenanthrene. Carcinogenesis 2: 1235–1239 (1981).
88. Miller, J. A., Sandin, R. B., Miller, E. C., and Rusch, H. P. The carcinogenicity of compounds related to 2-acetylaminofluorene. II. Variations in the bridges and the 2-substituent. Cancer Res. 15: 188–199 (1955).
89. Gupta, R. C., Earley, K., Fullerton, N. F., and Beland, F. A. Formation and removal of DNA adducts in rats administered multiple doses of 2-acetylaminophenanthrene. Proc. Am. Assoc. Cancer Res. 26: 86 (1985).
90. Beranek, D. T., Heflich, R. H., Morris, S. M., and Beland, F. A. Correlation between specific DNA adducts and mutation induction in Chinese hamster ovary cells exposed to *N*-hydroxy arylamines. J. Cell. Biochem. 8A: 45 (1984).
91. Koffel-Schwartz, N., Verdier, J.-M., Bichara, M., Freund, A.-M., Daune, M. P., and Fuchs, R. P. P. Carcinogen-induced mutation spectrum in wild-type, *uvrA* and *umuC* strains of *Escherichia coli*. J. Mol. Biol. 177: 33–51 (1984).
92. Wei, S.-J. C., Desai, S. M., Harvey, R. G., and Weiss, S. B. Use of short DNA oligonucleotides for determination of DNA sequence modification induced by benzo[a]pyrene diol epoxide. Proc. Natl. Acad. Sci. (U.S.) 81: 5936–5940 (1984).
93. Foster, P. L., Eisenstadt, E., and Miller, J. H. Base substitution mutations induced by metabolically activated aflatoxin B_1. Proc. Natl. Acad. Sci. (U.S.) 80: 2695–2698 (1983).
94. Moore, P. D., Rabkin, S. D., Osborn, A. L., King, C. M., and Strauss, B. S. Effect of acetylated and deacetylated 2-aminofluorene adducts on *in vitro* DNA synthesis. Proc. Natl. Acad. Sci. (U.S.) 79: 7166–7170 (1982).
95. Yoshida, S., Koiwai, O., Suzuki, R., and Tada, M. Arrest of DNA elongation by DNA polymerases by guanine adducts on 4-

hydroxyaminoquinoline 1-oxide-modified DNA template. Cancer. Res. 44: 1867–1870 (1984).
96. Kadlubar, F. F. A transversion mutation hypothesis for chemical carcinogenesis by N^2-substitution of guanine in DNA. Chem.-Biol. Interact. 31: 255–263 (1980).
97. Hingerty, B. E., and Broyde, S. Base displacement in AAF and AF modified dCpdG: *syn* and *anti* guanine. Internat. J. Quantum Chem. Quantum Biol. Symp. 9: 125–136 (1982).
98. Hingerty, B., and Broyde, S. AAF linked to the guanine amino group: a B-Z junction. Nucleic Acids Res. 11: 3241–3254 (1983).
99. Swenson, D. H., and Kadlubar, F. F. Properties of chemical mutagens and chemical carcinogens in relation to their mechanisms of action. In: Microbial Testers Probing Carcinogenesis, (I. C. Felkner, Ed.), Marcel Dekker, New York, 1981, pp. 3–33.
100. Fuchs, R. P. P. DNA binding spectrum of the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene significantly differs from the mutation spectrum. J. Mol. Biol. 177: 173–180 (1984).
101. Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R., and Chang, E. H. Mechanism of activation of a human oncogene. Nature 300: 143–149 (1982).
102. Reddy, E. R., Reynolds, R. K., Santos, E., and Barbacid, M. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature 300: 149–152 (1982).
103. Capon, D. J., Seeburg, P. H., McGrath, J. P., Hayflick, J. S., Edman, U., Levinson, A. D., and Goeddel, D. V. Activation of Ki-*RAS*[2] gene in human colon and lung carcinomas by two different point mutations. Nature 304: 507–513 (1983).
104. Sukumar, S., Notario, V., Martin-Zanca, D., and Barbacid, M. Induction of mammary carcinomas in rats by nitroso-methylurea involves malignant activation of H-*ras*-1 locus by single point mutations. Nature 306: 658–661 (1983).
105. Kraus, M. H., Yuasa, Y., and Aaronson, S. A. A position 12-activated H-*ras* oncogene in all HS578T mammary carcinosarcoma cells but not normal mammary cells of the same patient. Proc. Natl. Acad. (U.S.) 81: 5384–5388 (1984).
106. Yuasa, Y., Srivastava, S. K., Dunn, C. Y., Rhim, J. A., Reddy, E. P., and Aaronson, S. A. Acquisition of transforming properties by alternative point mutations with c-*bas/has* human proto-oncogene. Nature 303: 775–779 (1983).

EXHIBIT H

TOXICOLOGY AND APPLIED PHARMACOLOGY **139,** 203–211 (1996)
ARTICLE NO. 0159

Interaction with the Aromatic Hydrocarbon Receptor, CYP1A Induction, and Mutagenicity of a Series of Diaminotoluenes: Implications for Their Carcinogenicity

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The present study was undertaken to provide a rationale for the marked difference in carcinogenic potential among isomeric diaminotoluenes, in relation to their ability to induce their own bioactivation through CYP1A induction, their genotoxic potential, and their ability to bind to the cytosolic aromatic hydrocarbon (Ah) receptor. Of the four possible diaminotoluenes only the 2,3- and, to a lesser extent, the 2,4-isomer induced CYP1A activity. Similarly, only these two isomers could displace [^{3}H]T-CDD from the hepatic cytosolic Ah receptor. In the presence of Aroclor 1254-induced microsomes, 2,4- and 2,6-diaminotoluene were potent mutagens in the Ames test. Only 2,4-diaminotoluene could autoinduce its activation. Of the four isomers only 2,4-diaminotoluene is an established carcinogen and this is compatible with the present observations that it is the only isomer that stimulates its own activation through CYP1A induction, is metabolically converted to genotoxic intermediates, and binds to the Ah receptor. 2,6-Diaminotoluene is recognized as a mutagenic noncarcinogen and in the present studies it elicited a positive mutagenic response in the Ames test but failed to induce CYP1A activity and its own activation, and could not bind to the Ah receptor even at concentrations as high as 5×10^{-4} M. The present findings demonstrate that *in vitro* studies are very useful tools in predicting the carcinogenic potency of isomeric chemicals. © 1996 Academic Press, Inc.

The majority of chemicals that display carcinogenicity cause DNA damage which, if unrepaired, gives rise to a mutation and may lead to tumor development through complex, poorly understood mechanisms. In most cases, the chemical is unable to interact directly with DNA but may readily do so through metabolically formed reactive intermediates generated within the living organism. An unexplained paradox is that some chemicals which display clear *in vitro* mutagenicity in short-term assays, such as the Ames test, are devoid of carcinogenicity in rodent lifetime bioassays. Two mechanisms may be proposed to rationalize such observations. The first may be related to the potential of these chemicals to induce the enzymes, primarily cytochrome P450 proteins, that catalyze their activation and thus enhance the generation of genotoxic intermediates (Ioannides, 1990). The cytochrome P450 subfamily most closely associated with the activation of planar carcinogens is CYP1A (Gueng-

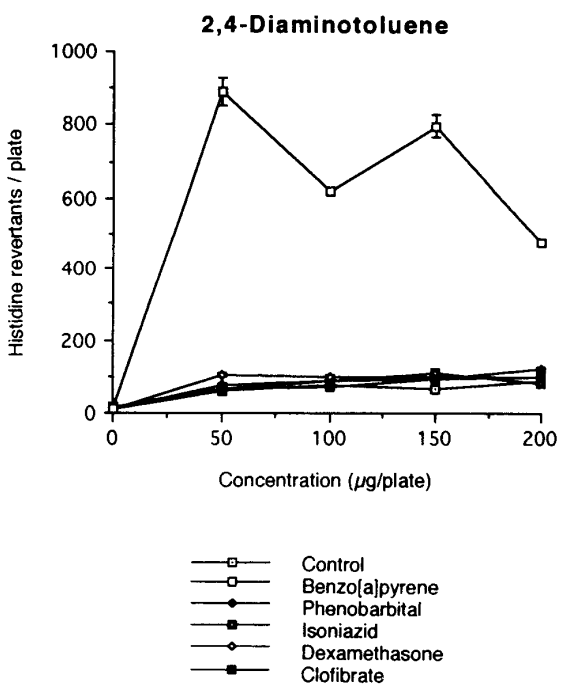

FIG. 1. Effect of cytochrome P450 inducers on the mutagenicity of 2,4-diaminotoluene in the Ames test. Mutagenicity was determined using *Salmonella typhimurium* strain TA98 and hepatic microsomes (25%, w/v) from rats pretreated with various inducing agents as activation systems at a final concentration of 10% (v/v). Plates were supplemented with 1 unit glucose 6-phosphate dehydrogenase. Results are expressed as mean ± SEM of triplicates.

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TABLE 1
The Effect of Diaminotoluenes on Rat Hepatic Microsomal Cytrochrome Content

Treatment	Dose (mg/kg)	Cytochrome b_5 (nmol/mg protein)	Cytochrome P450 (nmol/mg protein)	Microsomal protein (mg/g liver)
Control		0.59 ± 0.05	0.65 ± 0.03	18 ± 1
2,3-Diaminotoluene	10	0.70 ± 0.04	0.56 ± 0.07	18 ± 1
	20	0.63 ± 0.01	0.63 ± 0.01	19 ± 2
	40	0.62 ± 0.04	0.56 ± 0.02	21 ± 1
2,5-Diaminotoluene	10	0.63 ± 0.03	0.54 ± 0.06	22 ± 2
	20	0.62 ± 0.02	0.47 ± 0.06	22 ± 1
	40	0.63 ± 0.02	0.49 ± 0.04	25 ± 1**
Control		0.37 ± 0.02	0.31 ± 0.04	27 ± 1
2,4-Diaminotoluene	10	0.43 ± 0.01*	0.34 ± 0.04	22 ± 1*
	20	0.44 ± 0.03	0.51 ± 0.06*	22 ± 2
	40	0.40 ± 0.02	0.40 ± 0.03	24 ± 1
Control		0.60 ± 0.07	0.32 ± 0.02	38 ± 1
2,6-Diaminotoluene	10	0.60 ± 0.02	0.33 ± 0.03	44 ± 2
	20	0.50 ± 0.04	0.31 ± 0.01	39 ± 1
	40	0.73 ± 0.11	0.35 ± 0.01	36 ± 2

Note. Animals received a single intraperitoneal dose of the chemical and were killed 24 hr following the injection. Results are expressed as mean ± SEM of four animals.
*$p < 0.05$.
**$p < 0.01$.

erich and Shimada, 1991; Gonzalez and Gelboin, 1994; Ioannides and Parke, 1990) whose basal levels are very low but are readily and markedly inducible by these carcinogens (Parke and Ioannides, 1994). The induction of CYP1A proteins is regulated by a cytosolic receptor protein, namely the Ah receptor (Nebert and Jones, 1989). It is conceivable that a chemical which displays mutagenicity in the Ames test, where activation is catalyzed by a hepatic system with artificially high CYP1A activity as a result of induction with Aroclor 1254, fails to elicit a positive carcinogenic response in rodent lifetime bioassays if it is unable to induce this enzyme system and has to rely on the basal levels for its activation. Indeed, we have reported good correlations between the ability of various groups of chemical carcinogens to induce CYP1A activity and carcinogenic potential (Ayrton *et al.,* 1990a,b; Ioannides *et al.,* 1993; Cheung *et al.,* 1994). A second, alternative explanation is that an *in vitro* genotoxin is rapidly excreted *in vivo,* e.g., by having a functional group(s) that readily forms a conjugate.

The diaminotoluenes are synthetic chemicals used in the manufacture of polyurethane foam and dyes. The four possible isomers differ in their carcinogenic potential. 2,4-Diaminotoluene has been described as a moderate carcinogen inducing tumors in the livers of both rats and mice (Ito *et al.,* 1969; Cardy, 1979; NCI, 1979). The 2,5-isomer was tested for carcinogenicity only as a component of hair dyes which also included the carcinogenic 2,4-diaminotoluene and, consequently, its carcinogenic potential cannot be evaluated. In the Ames test, in the presence of an activation system, it provoked a positive mutagenic response (Furlong *et al.,* 1987). 2,6-Diaminotoluene is classified as a noncarcinogen, having failed to induce tumors in both rats and mice (NCI, 1980), but in the Ames test, in the presence of an activation system, it elicits a mutagenic response (Furlong *et al.,* 1987). No data are available on either the mutagenicity or the carcinogenicity of 2,3-diaminotoluene.

The aim of the present study is to provide a rationale for the difference in carcinogenic potential among the diaminotoluenes on the basis of (a) their genotoxic potential as exemplified by the mutagenic response in the Ames test, (b) induction of the enzyme system that catalyzes their activation, and (c) interaction with the Ah receptor which, in addition to CYP1A induction, also regulates the genes involved in cellular proliferation, which is an important property of complete carcinogens.

MATERIALS AND METHODS

Diaminotoluenes (Aldrich Chemicals, Gillingham, Dorset), resorufin and alkoxyresorufins (Molecular Probes, Eugene, OR, USA), Aroclor 1254 (Robens Institute, University of Surrey), 2,3,7,8-tetrachloro-*p*-dioxin (TCDD) (Chemsyn Laboratories, Kansas, USA.), 2,3,7,8-tetrachloro-dibenzofuran (TCDF) (Promochem, St. Albans, Herts), benzo[*a*]pyrene, isoniazid, clofibrate, dexamethasone, horseradish peroxidase-labeled anti-sheep antibody, and all cofactors (Sigma Co., Poole, Dorset) were all purchased. The Salmonella typhimurium strain TA98 was a kind gift from Professor B. N. Ames (Berkeley, CA). The purification and characterization of CYP1A1 from male Wistar albino rats has already been described (Rodrigues *et al.,* 1987). The antibodies raised in sheep could recognize both CYP1A1 and CYP1A2 proteins.

Male Wistar albino rats (150–200 g) were purchased from the Experimental Biology Unit, University of Surrey. Animals were given a single intraperitoneal injection of the diaminotoluene at three dose levels, 10, 20, or 40 mg/kg, whereas controls received the corresponding volume of corn oil, the vehicle. All animals were killed 24 hr after administration. For the

selective induction of cytochrome P450 proteins, the animals received single daily intraperitoneal administrations of benzo[*a*]pyrene (25 mg/kg), phenobarbitone (80 mg/kg), or clofibrate (80 mg/kg) or by gastric intubation isoniazid (100 mg/kg) or dexamethasone (100 mg/kg). Treatment was carried out for 3 days, all animals being killed 24 hr after the last administration. Finally, in the case of Aroclor 1254, animals were given a single intraperitoneal dose (500 mg/kg) and were killed on the 5th day following administration. The animals were maintained at 45–55% humidity and 23°C throughout the study period. A 12-hr light/dark cycle was in operation.

Hepatic microsomal and cytosolic fractions were prepared as previously described (Ioannides and Parke, 1975), and the following assays were performed using the microsomal fraction: the *O*-dealkylations of ethoxy- (Burke and Mayer, 1974) and methoxyresorufin (Burke and Mayer, 1983), *N*-hydroxylation of the diaminotoluenes (Vanderslice *et al.,* 1987), cytochrome b5 and total cytochrome P450 (Omura and Sato, 1964), and protein (Lowry *et al.,* 1957). Immunoblot analysis of the solubilized microsomal proteins following resolution by electrophoresis (Laemmli, 1970) was carried out as described by Towbin *et al.* (1979). Binding to the cytosolic Ah receptor was determined using the hydroxyapatite method (Gasiewicz and Neal, 1982).

The mutagenic potential of the diaminotoluenes was assayed using the Ames test (Maron and Ames, 1983), employing *Salmonella typhimurium* strain TA98 and activation systems containing 10% (v/v) of the microsomal fraction (25%, w/v). The activation system was supplemented with glucose 6-phosphate dehydrogenase (1 unit/plate). A 30-min preincubation test was incorporated to allow the generation of the mutagenic species. All diaminotoluenes were dissolved in dimethyl sulphoxide so that the volume never exceeded 100 μl/plate.

Statistical evaluation was carried out using the Student's *t* test.

RESULTS

2,4-Diaminotoluene displayed a positive mutagenic response in the Ames test only in the presence of an activation system derived from rats pretreated with benzo[*a*]pyrene (Fig. 1). When the activation system was isolated from rats pretreated with the other cytochrome P450 inducers or from untreated animals, the mutagenic response was relatively weak.

Rats treated with the various diaminotoluene isomers did not modulate cytochrome b_5 or total cytochrome P450 levels in a dose-dependent way (Table 1). As we have already reported (Cheung *et al.,* 1993, 1994), there were considerable differences in basal levels between the various animal batches. Treatment with 2,3-diaminotoluene gave rise to a dose-dependent increase in the hepatic *O*-deethylation of ethoxyresorufin. At the highest dose a statistically significant, but less pronounced, increase was observed in the *O*-demethylation of methoxyresorufin (Fig. 2). Similar treatment with 2,4-diaminotoluene resulted in a relatively modest increase in the *O*-deethylation of ethoxyresorufin. None of the other diaminotoluene isomers influenced either of these activities. Immunoblot analysis using antibodies to CYP1A revealed that treatment with 2,3-diaminotoluene caused a dose-dependent increase in the apoprotein levels of CYP1A1 and CYP1A2, the former not being detectable in the control animals (Fig. 3). A similar, but less pronounced effect, was seen with 2,4-diaminotoluene but no such effect was observed with the remaining two isomers.

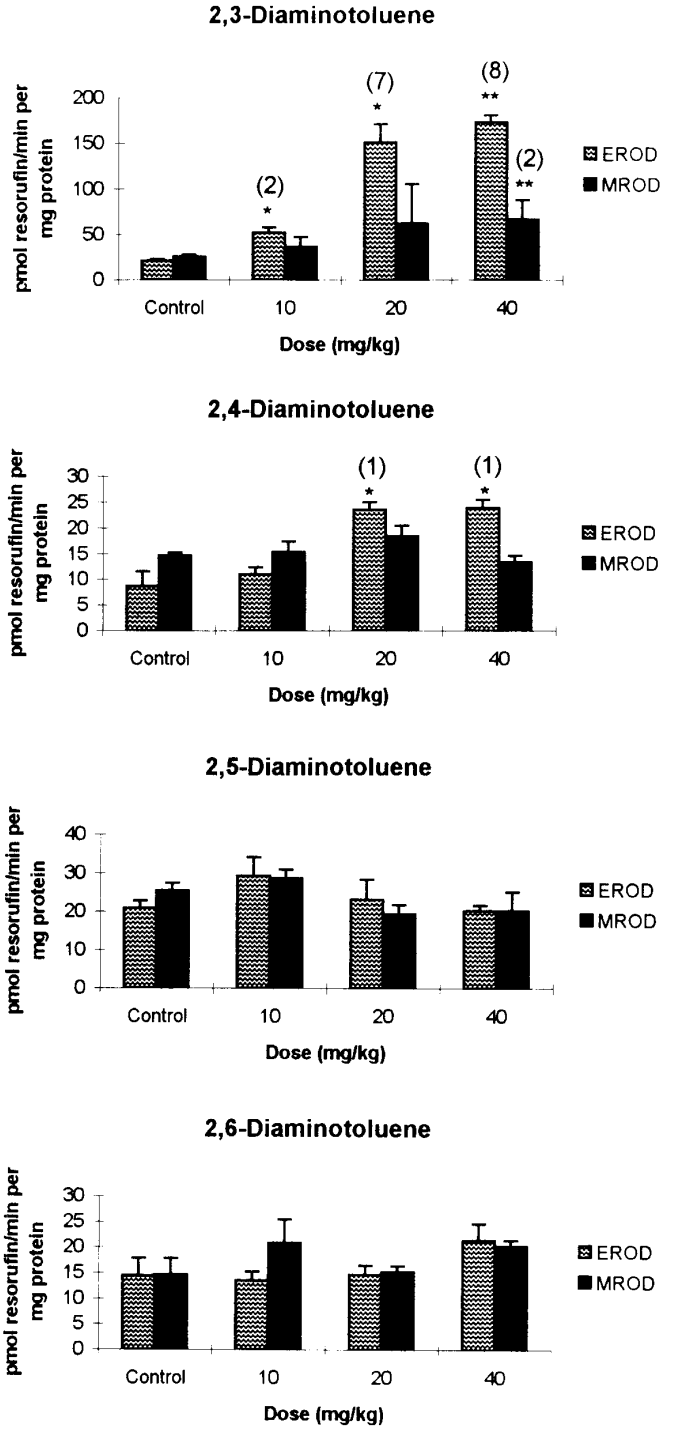

FIG. 2. The effect of diaminotoluenes on the hepatic *O*-dealkylations of methoxy- and ethoxyresorufin in the rat. Rats received a single intraperitoneal dose of the chemical and were killed 24 hr after the administration. Results are expressed as mean ± SEM of four animals. Numbers in parentheses represent fold induction. $*p < 0.05$; $**p < 0.01$.

2,3-Diaminotoluene and, to a lesser extent, the 2,4-isomer displaced TCDD from the hepatic cytosolic Ah receptor, whereas the 2,5- and 2,6-isomers could not do so at concen-

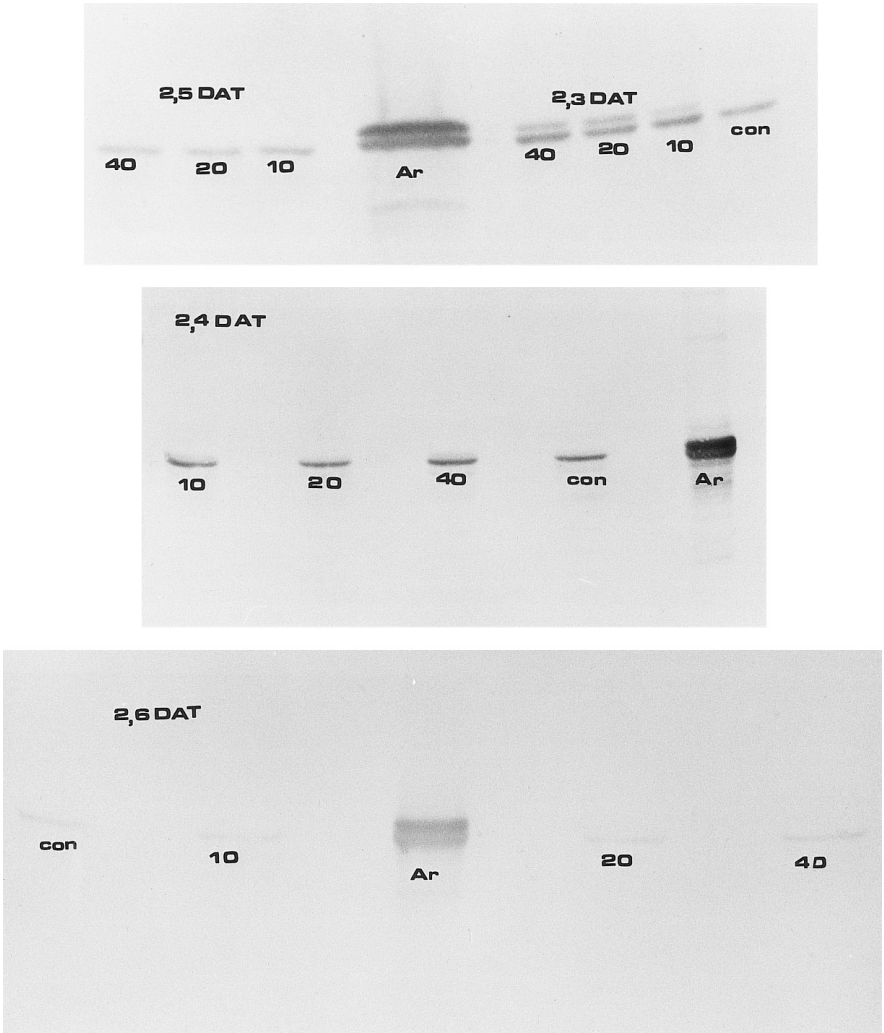

FIG. 3. Immunoblot analysis of hepatic microsomes from diaminotoluene-treated rats probed with anti-CYP1A1 antibodies. Microsomal proteins (20 μg) were resolved by electrophoresis in a 10% (w/v) SDS–polyacrylamide gel and transferred electrophoretically onto nitrocellulose. The immunoblot was carried out with sheep anti-CYP1A1 (diluted 1:10,000) followed by peroxidase-labeled anti-sheep IgG (diluted 1:2500). con, control; DAT, diaminotoluene; Ar, Aroclor 1254. Of the two recognized bands, the top is CYP1A1 and the bottom CYP1A2.

trations as high as 5×10^{-4} M (Fig. 4). The EC_{50} values are shown in Table 2.

2,3-Diaminotoluene displayed weak mutagenic response in the Ames test in the presence of hepatic microsomes from untreated rats; mutagenicity was not significantly altered by pretreatment of the animals with either Aroclor 1254 or the diaminotoluene itself (Fig. 5). In the presence of hepatic microsomes from untreated rats, 2,4-diaminotoluene exhibited weak mutagenicity which was, however, markedly induced following treatment with Aroclor 1254. Increased mutagenicity was also evident when the source of the activation system was 2,4-diaminotoluene-treated rats (Fig. 5). 2,5-Diaminotoluene displayed weak mutagenicity but only when Aroclor 1254-induced hepatic microsomes served as the activation system. Finally, 2,6-diaminotoluene elicited marked mutagenic response only in the presence of an activation system from Aroclor 1254-induced animals (Fig. 5).

Hepatic microsomes from control rats could *N*-hydroxylate all diaminotoluene isomers but to a varying extent, the 2,5-isomer being the best substrate. Pretreatment with Aroclor 1254 enhanced the *N*-hydroxylation of all isomeric diaminotoluenes by some two- to threefold (Table 3). The *N*-hydroxylation of 2-aminofluorene, used as a positive control, was the most extensively induced by the treatment with Aroclor 1254.

DISCUSSION

For a compound to display carcinogenicity at least two prerequisites must be fulfilled (Ioannides, 1990): it must be or have the potential to be metabolically converted to a reactive intermediate(s) capable of interaction with DNA and, second, the living organism, at the time of exposure, must possess the necessary enzymic systems that will allow

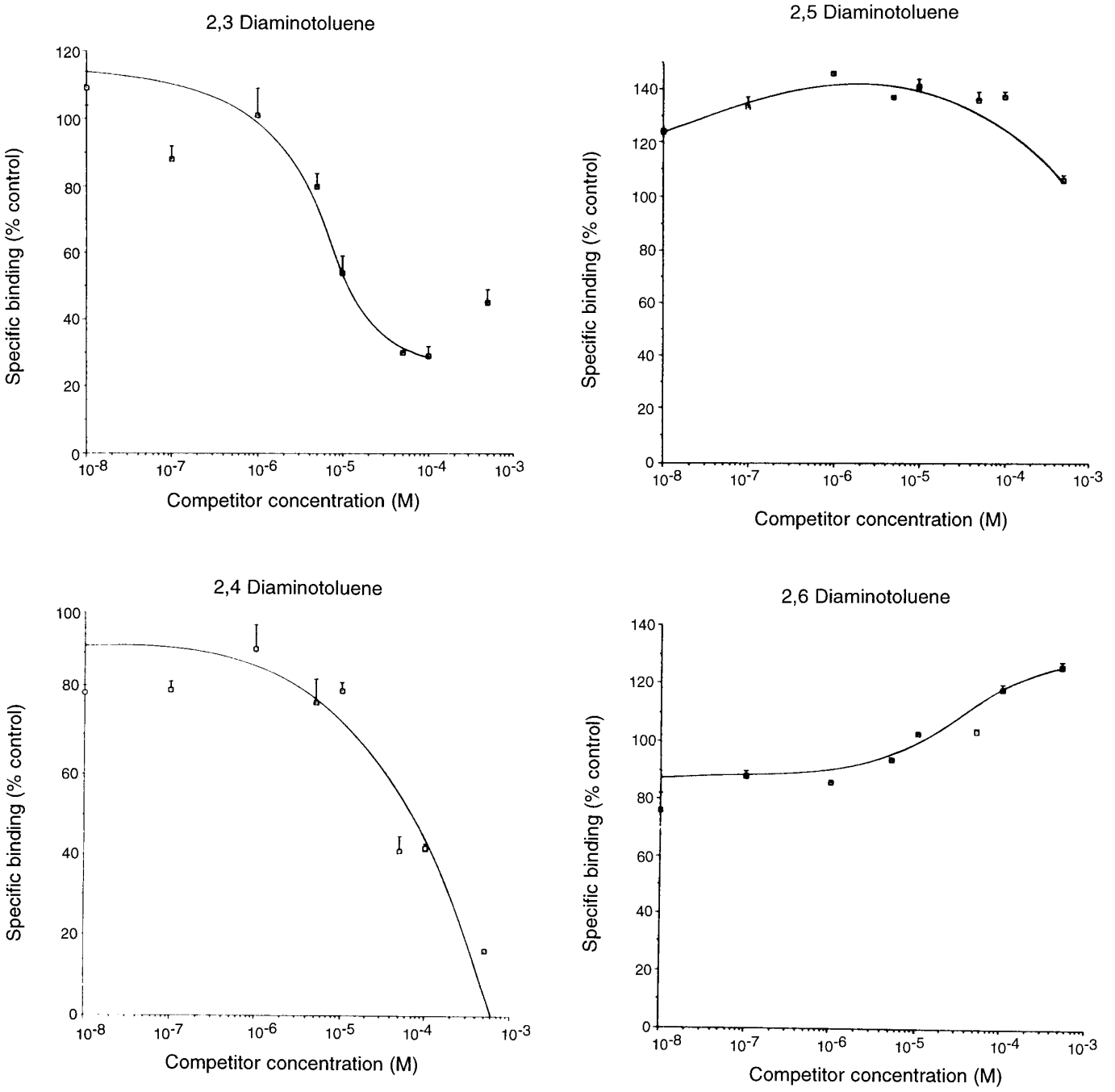

FIG. 4. Displacement of [^{3}H]TCDD from the rat hepatic cytosolic Ah receptor by diaminotoluenes. The assay was performed using [^{3}H]TCDD (0.3 nM) as the ligand and a protein concentration of 2 mg/ml. Nonspecific binding was calculated from incubations of the ligand with TCDF (200 nM). Each point represents the mean of three determinations.

the generation of this intermediate(s). In cases where the enzymes responsible for bioactivation are constitutively poorly expressed, the chemical in question may, on repeated administration, selectively induce these enzymes, thus stimulating its own activation (Ioannides and Parke, 1994). For example, CYP1A1, which plays a major role in the bioactivation of polycyclic aromatic hydrocarbons (Bauer *et al.*, 1995), is also markedly induced by these compounds (Ayrton *et al.*, 1990b; Cheung *et al.*, 1993).

The bioactivation of aromatic amines proceeds through *N*-hydroxylation, catalyzed by the cytochrome P450-dependent mixed-function oxidases and, to a lesser extent, by the flavin monooxygenases. Ring-hydroxylation and *N*-acetylation are usually deactivation pathways. Indeed, these pathways of

TABLE 2
Displacement of [^{3}H]TCCD from the Rat Hepatic Cytosolic Ah Receptor by Diaminotoluenes

Compound	Displacement of [^{3}H]TCCD EC_{50} (M)
2,3-Diaminotoluene	1.5×10^{-5}
2,4-Diaminotoluene	7.0×10^{-5}
2,5-Diaminotoluene	No displacement
2,6-Diaminotoluene	No displacement

Note. At least seven concentrations of each displacer have been employed, each being performed in triplicate.

metabolism have been shown to be involved in 2,4-diaminotoluene (Cunningham and Matthews, 1990; Waring and Pheasant, 1976; Grantham *et al.,* 1980) and 2,6-diaminotoluene (Cunningham *et al.,* 1989). Of the two amino groups, *N*-hydroxylation at the 2-position appears not to be favored, presumably as a result of steric hindrance by the methyl group. In the case of the 2,4-isomer, experimental evidence points to the 4-position as the site for *N*-hydroxylation (Cunningham and Matthews, 1990). This concept is in complete agreement with our present findings that *N*-hydroxylation occurred markedly more readily with the 2,4- and 2,5-diaminotoluenes, with both control and Aroclor 1254-treated microsomes, i.e., where the adjacent positions to the amino group are unoccupied.

An initial study was undertaken to identify the inducible cytochrome P450 protein(s) that participate in the *N*-hydroxylation, and consequently the activation of the diaminotoluenes. Using 2,4-diaminotoluene as the model, its conversion to mutagens in the Ames test was determined in the presence of microsomal activation systems isolated from the liver of rats treated with selective inducers of cytochrome P450 proteins. Only treatment with benzo[*a*]pyrene, an inducer of CYP1A, caused a marked increase in mutagenic response, whereas all other treatments had no significant effect. These results indicate that, among the inducible isoforms of cytochrome P450, only CYP1A catalyzes the bioactivation of 2,4-diaminotoluene, in concert with our findings that treatment with Aroclor 1254, a very potent inducer of the CYP1A subfamily (see Fig. 3), enhanced the *N*-hydroxylation of this amine. Similarly, in studies where postmitochondrial preparations, rather than isolated microsomes, served as activation systems, treatment with the CYP1A-specific inducer β-naphthoflavone resulted in increased mutagenicity of 2,4-diaminotoluene in the Ames test (Furlong *et al.,* 1987). The same workers also reported that the same treatment enhanced the mutagenic effect of the 2,5- and 2,6-isomers. Collectively the above data provide strong experimental evidence that a major catalyst of the activation of diaminotoluenes is the CYP1A subfamily. This is not surprising bearing in mind that substrates of the CYP1 family are essentially planar molecules (Lewis *et al.,* 1986, 1993). However, the possibility that other, noninducible cytochrome P450 proteins or the flavin monooxygenase system make a contribution to the activation of these amines cannot be ruled out.

Of the four diaminotoluene isomers, only the 2,3-isomer was an inducer of CYP1A activity, as exemplified by the *O*-dealkylations of methoxy- and ethoxyresorufin, probes for the CYP1A1 and CYP1A2, respectively (Namkung *et al.,* 1988). A much weaker effect was observed with the 2,4-diaminotoluene but the other isomers did not influence CYP1A activity. Immunological work confirmed these findings and established unequivocally that both A1 and A2 isoforms of the CYP1 family were induced. The fact that only the 2,3- and 2,4-diaminotoluenes interacted with the Ah receptor indicates that their ability to induce CYP1A may be regulated, at least partly, by this receptor. Furthermore, 2,3-diaminotoluene, the more potent CYP1A inducer, bound to the receptor more avidly than the 2,4-isomer, as judged by their ability to displace TCCD. Diaminotoluenes are relatively weak inducers of CYP1A compared to polycyclic aromatic hydrocarbons and this is in agreement with the observation that the latter compounds bind to the Ah receptor much more avidly, having EC_{50} values around 10^{-9} M (Cheung *et al.,* 1993), compared to 10^{-5} M for the diaminotoluenes.

Of the isomers studied only the 2,4-diaminotoluene has been described as a carcinogen following long-term feeding studies in the rat and mouse, inducing hepatocellular carcinomas (Ito *et al.,* 1969; NCI, 1979) and this could have been predicted from the present *in vitro* studies. 2,4-Diaminotoluene is (a) readily *N*-hydroxylated by hepatic microsomes, (b) the resulting hydroxylamine is genotoxic as evidenced by the positive response in the Ames test, (c) on repeated administration it self-induces its bioactivation, and (d) it interacts with the Ah receptor, thus causing cellular proliferation and promotion of the mutated cells. Indeed, in studies performed in the rat, 2,4-diaminotoluene administration gave rise to a dose-dependent increase in hepatic cellular proliferation (Cunningham *et al.,* 1991). In contrast to the 2,4-isomer, 2,6-diaminotoluene in similar feeding studies failed to induce tumors in either rats or mice (NCI, 1980) and is considered as a genotoxic noncarcinogen. Its mutagenicity, less pronounced than that of the 2,4-isoform, was confirmed in the present study using isolated microsomes rather than postmitochondrial supernatant as in previous studies (Cunningham *et al.,* 1989; Furlong *et al.,* 1987). Bearing in mind the poor rate of *N*-hydroxylation of this compound, it may be inferred that its ultimate carcinogen, presumably the nitrenium ion, is highly genotoxic. However, 2,6-diaminotoluene does not induce CYP1A activity and its own activation. In addition, it does not interact with the Ah receptor and thus it is unlikely to function as a tissue proliferator. Indeed, this isomer, in contrast to 2,4-diaminotoluene, failed to

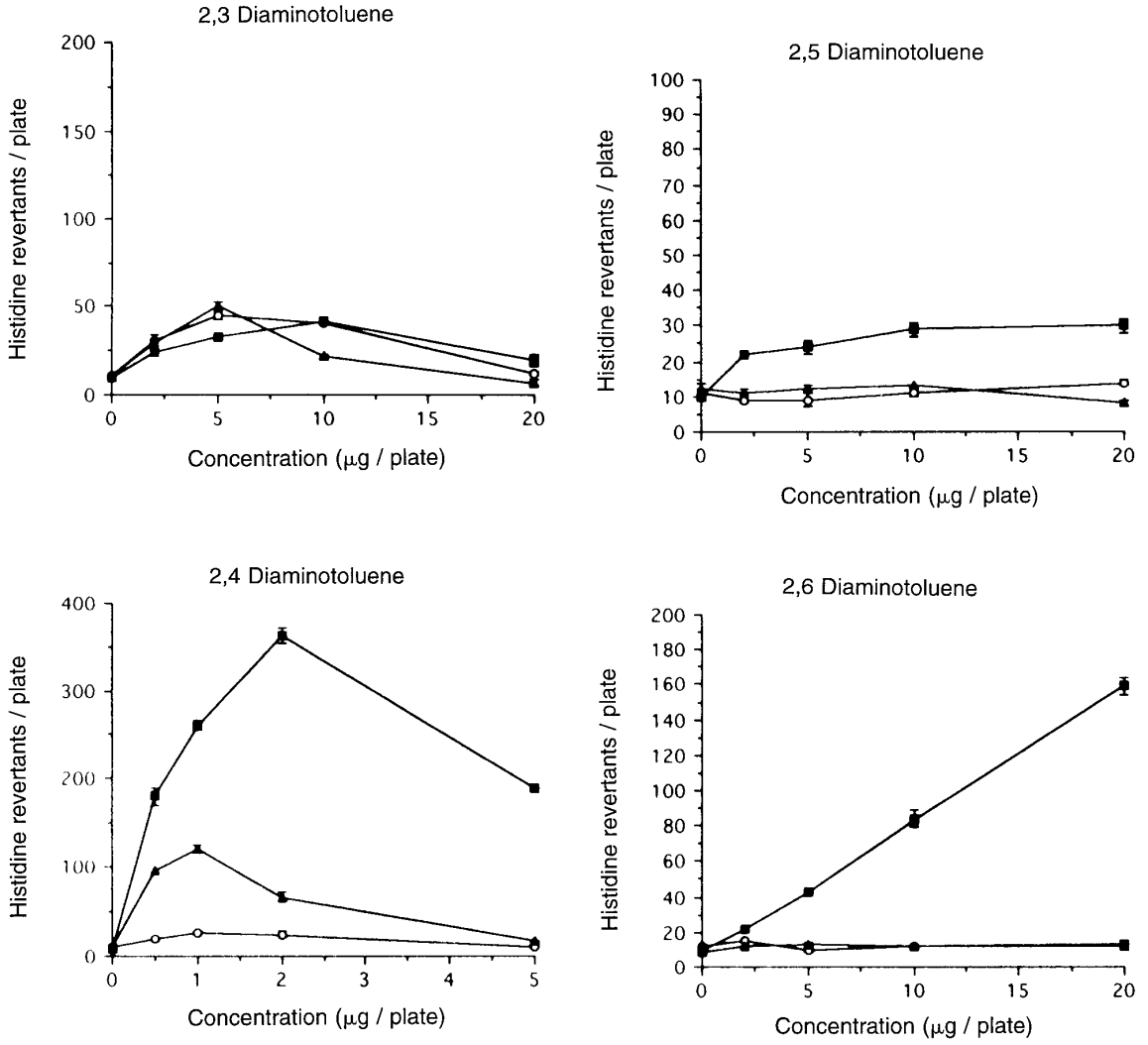

FIG. 5. Mutagenicity of diaminotoluenes in the Ames test. The test was carried out using *Salmonella typhimurium* strain TA98 and hepatic microsomes (25%, g/v), at a final concentration of 10% (v/v), derived from control (○), diaminotoluene-treated (▲), and Aroclor 1254-treated rats (■). Plates were supplemented with 1 unit of glucose 6-phosphate dehydrogenase. Activation system, mutagen, and bacteria were preincubated for 30 min in a shaking water bath at 37°C. Results are expressed as mean ± SEM of triplicates. The study was repeated using samples from other, but similarly treated, animals with similar results.

increase hepatic cell turnover when administered to rats (Cunningham *et al.*, 1991). These observations may explain why 2,6-diaminotoluene is a mutagen but not a full carcinogen. An additional consideration is that it may be rapidly deactivated through ring hydroxylations and *N*-acetylation. It is, however, pertinent to point out that the metabolite of 2,6-diaminotoluene where both amino groups had undergone acetylation retained their mutagenic activity (Cunningham *et al.*, 1989). 2,3-Diaminotoluene has a minimal genotoxic potential, possibly reflecting its poor *N*-hydroxylation, it does not induce its own activation, and consequently it is unlikely to be a carcinogen per se. However, it induces CYP1A activity and may act as a cocarcinogen by potentiating the effect of carcinogens that rely on this subfamily for their activation. It also binds to the Ah receptor and so may function as a promoter of mutated cells. 2,5-Diaminotoluene is a weak mutagen despite its high rate of *N*-hydroxylation. A higher mutagenicity has been described in the presence of β-naphthoflavone-induced postmitochondrial supernatant (Furlong *et al.*, 1987). However, as it does not stimulate its own activation and does not bind to the Ah receptor, our prediction is that this isomer is unlikely to be a carcinogen.

The present study illustrates how *in vitro* studies, based on the understanding of the pathways of activation, of the enzymes catalyzing these and genotoxic potential, enable the prediction of the carcinogenic potential of a given chemical. Moreover, such studies are relatively inexpensive and can be completed in a matter of weeks. This approach helps to ensure that the long-term and highly

TABLE 3
The *N*-Hydroxylation of Diaminotoluenes by Control and Aroclor 1254-Treated Rat Hepatic Microsomes

Compound	Control[a]	Aroclor 1254-induced[a]
2,3-Diaminotoluene	61 ± 2	114 ± 7*
2,4-Diaminotoluene	429 ± 9	1273 ± 40*
2,5-Diaminotoluene	1323 ± 23	2784 ± 143*
2,6-Diaminotoluene	60 ± 3	167 ± 6*
2-Aminofluorene	320 ± 3	1798 ± 103*

Note. Results are presented as mean ± SEM of three animals.
[a] *N*-Hydroxylation activities are expressed as nmol/min per mg protein.
* $p < 0.001$.

expensive carcinogenicity studies are not conducted needlessly.

ACKNOWLEDGMENT

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REFERENCES

Ayrton, A. D., McFarlane, M., Walker, R., Neville, S., and Ioannides, C. (1990a). The induction of P450I proteins by aromatic amines may be related to their carcinogenic potential. *Carcinogenesis* **11,** 803–809.

Ayrton, A. D., McFarlane, M., Walker, R., Neville, S., Coombs, M. M., and Ioannides, C. (1990b). Induction of the P450I family of proteins by polycyclic aromatic hydrocarbons: Possible relationship to their carcinogenicity. *Toxicology* **60,** 173–186.

Bauer, E., Guo, Z., Yeng, Y-F., Bell, C., Zeldin, D., and Guengerich, F. P. (1995). Oxidation of benzo(a)pyrene by recombinant human cytochrome P450 enzymes. *Chem. Res. Toxicol.* **8,** 136–142.

Burke, M. D., and Mayer, R. T. (1974). Ethoxyresorufin; direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Dispos.* **2,** 583–588.

Burke, M. D., and Mayer, R. T. (1983). Differential effects of phenobarbitone and 3-methylcholanthrene induction on the hepatic microsomal and cytochrome P450 binding of phenoxazone and a homologous series of its n-alkyl ethers (alkoxyresorufins). *Chem. Biol. Interactions* **45,** 243–258.

Cardy, R. H. (1979). Carcinogenicity and chronic toxicity of 2,4-toluenediamine in F344 rats. *J. Natl. Cancer Inst.* **62,** 1107–1116.

Cheung, Y-L., Gray, T. J. B., and Ioannides, C. (1993). Mutagenicity of chrysene, its methyl and benzo derivatives, and their interactions with cytochromes P450 and the Ah-receptor: Relevance to their carcinogenic potency. *Toxicology* **81,** 69–86.

Cheung, Y-L., Puddicombe, S. M., Gray, T. J. B., and Ioannides, C. (1994). Mutagenicity and CYP1A induction by azobenzenes correlates with their carcinogenicity. *Carcinogenesis* **15,** 1257–1263.

Cunningham, M. L., Burka, L. T., and Matthews, H. B. (1989). Metabolism disposition, and mutagenicity of 2,6-diaminotoluene, a mutagenic noncarcinogen. *Drug Metab. Dispos.* **17,** 612–617.

Cunningham, M. L., and Matthews, H. B. (1990). Evidence for an acetoxyarylamine as the ultimate mutagenic reactive intermediate of the carcinogenic aromatic amine 2,4-diaminotoluene. *Mutat. Res.* **242,** 101–110.

Cunningham, M. L., Foley, J., Maronpot, R. R., and Matthews, H. B. (1991). Correlation of hepatocellular proliferation with hepatocarcinogenicity induced by the mutagenic noncarcinogen:carcinogen pair—2,6- and 2,4-diaminotoluene. *Toxicol. Appl. Toxicol.* **107,** 562–567.

Furlong, B. B., Weaver, R. P., and Goldstein, J. A. (1987). Covalent binding to DNA and mutagenicity of 2,4-diaminotoluene metabolites produced by isolated hepatocytes and 9000g supernatant from Fisher 344 rats. *Carcinogenesis* **8,** 247–251.

Gasiewicz, T. A., and Neal, R. A. (1982). The examination and quantitation of tissue cytosolic receptors for 2,3,7,8-tetrachlorobenzo-p-dioxin using hydroxylapatite. *Anal. Biochem.* **124,** 1–11.

Gonzalez, F. J., and Gelboin, H. V. (1994). Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metab. Rev.* **26,** 165–183.

Gonzalez, F. J., and Shimada, T. (1994). Role of human cytochromes P450 in the activation of chemical carcinogens and toxins. *Drug Metab. Rev.* **26,** 165–183.

Grantham, P. H., Mohan, L., Benjamin, T., Roller, P. P., Miller, J. R., and Weisburger, E. K. (1980). Comparison of the metabolism of 2,4-toluenediamine in rats and mice. *J. Environ. Pathol. Toxicol.* **3,** 149–166.

Guengerich, F. B., and Shimada, T. (1991). Oxidation of toxic and carcinogenic chemicals by human cytochrome P450 enzymes. *Chem. Res. Toxicol.* **4,** 391–407.

Ioannides, C. (1990). Induction and its influence on chemical carcinogenesis. *Biochem. Soc. Trans.* **18,** 32–34.

Ioannides, C., Cheung, Y-L., Wilson, J., Lewis, D. F. V., and Gray, T. J. B. (1993). The mutagenicity and interactions of 2- and 4-acetylaminofluorene with cytochrome P450 and aromatic hydrocarbon receptor may explain the difference in their carcinogenic potency. *Chem. Res. Toxicol.* **6,** 525–541.

Ioannides, C., and Parke, D. V. (1975). Mechanism of induction of hepatic drug metabolising enzymes by a series of barbiturates. *J. Pharm. Pharmacol.* **27,** 739–749.

Ioannides, C., and Parke, D. V. (1990). The cytochrome P450I gene family of microsomal haemoproteins and their role in the metabolic activation of chemicals. *Drug Metab. Rev.* **22,** 1–85.

Ioannides, C., and Parke, D. V. (1994). Induction of cytochrome P4501 as an indicator of potential chemical carcinogenesis. *Drug Metab. Rev.* **25,** 485–501.

Ito, D., Hiasa, Y., Konishi, Y., and Marugami, M. (1969). The development of carcinoma in liver of rats treated with m-toluenediamine and the synergistic and antagonistic effects of other chemicals. *Cancer Res.* **29,** 1137–1145.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227,** 680–685.

Lewis, D. F. V., Ioannides, C., and Parke, D. V. (1986). Molecular dimensions of the substrate binding site of cytochrome P448. *Biochem. Pharmacol.* **35,** 2179–2185.

Lewis, D. F. V., Ioannides, C., and Parke, D. V. (1993). Validation of a novel molecular orbital approach (COMPACT) for the prospective safety evaluation of chemicals by comparison with rodent carcinogenicity and Salmonella mutagenicity data evaluated by the U.S. NC1/NTP. *Mutat. Res.* **291,** 61–77.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, A. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–271.

Maron, D. M., and Ames, B. N. (1983). Revised methods for the Salmonella mutagenicity test. *Mutat. Res.* **113,** 173–215.

Namkung, M. J., Yang, H. L., Hulla, J. E., and Juchau, M. R. (1988). On the substrate specificity of P450IIIA1. *Mol. Pharmacol.* **34,** 628–637.

National Cancer Institute (NCI) (1979). Bioassay of 2,4-diaminotoluene for possible carcinogenicity. *Technical Report Series,* No. 162.

National Cancer Institute (NCI) (1980). Bioassay of 2,6-toluenediamine dihydrochloride for possible carcinogenicity. *Technical Report Series*. No. 200.

Nebert, D. W., and Jones, J. E. (1989). Regulation of the mammalian cytochrome P450 (CYP1A1) gene. *Int. J. Biochem.* **21,** 243–252.

Nerurkar, P. V., Park, S. S., Thomas, P. E., Nims, R. W., and Lubet, R. A. (1993). Methoxyresorufin and benzyloxyresorufin: Substrates preferentially metabolised by cytochromes P4501A2 and 2B, respectively in the rat and mouse. *Biochem. Pharmacol.* **46,** 933–943.

Omura, T., and Sato, R. (1964). The carbon monoxide pigment of liver microsomes. I. Evidence for its haemoprotein nature. *J. Biol. Chem.* **239,** 2370–2378.

Rodrigues, A. D., Gibson, G. G., Ioannides, C., and Parke, D. V. (1987). Interactions of imidazole antifungal agents with purified cytochrome P450 proteins. *Biochem. Pharmacol.* **36,** 4277–4281.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels with nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4355.

Vanderslice, R. R., Domin, B. A., Carver, G. T., and Philpot, R. M. (1987). Species-dependent expression and induction of homologues of rabbit cytochrome P450 isozyme 5 in liver and lung. *Mol. Pharmacol.* **31,** 320–325.

Waring, R. H., and Pheasant, A. E. (1976). Some phenolic metabolites of 2,4-diaminotoluene in the rabbit, rat and guinea pig. *Xenobiotica* **6,** 257–262.

EXHIBIT I

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TOXICOLOGY

Genotoxic and non-genotoxic activities of 2,4- and 2,6-diaminotoluene, as evaluated in Fischer-344 rat liver

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Abstract

Among aminoaromatics, 2,4-diaminotoluene (2,4-DAT) and 2,6-diaminotoluene (2,6-DAT) represent a conflicting couple of isomers; despite showing the same structural alert to DNA reactivity (and thus potential genotoxicity), they are different in terms of carcinogenicity. Of the two, 2,4-DAT alone is a potent rodent carcinogen, the liver being its major target. According to the literature, assays using various short-term genotoxicity tests have not discriminated satisfactorily between the carcinogenic and non-carcinogenic isomer, both chemicals producing overall positive results. To investigate their mechanism of action, we assayed both 2,4-DAT and 2,6-DAT in F-344 rat liver for their ability to induce DNA adducts, as detected by the ^{32}P-postlabelling technique, and to enhance the induction of preneoplastic foci, as detected by GGT-staining in diethylnitrosamine (DENA)-initiated hepatocytes. Our expectation was that, using the correct target/metabolism, a classic genotoxicity assay and an assay detecting non-genotoxic activities could, together, reflect the different carcinogenic behaviour of the two isomers. The results indicate that, at the single equimolar dose of 250 mg/kg i.p., 2,4-DAT was able to induce ~6500 times more DNA adducts than 2,6-DAT; the estimated RAL values for the two isomers were 18.6×10^{-6} and 0.29×10^{-8}, respectively. Moreover, of the two, only 2,4-DAT was able to significantly enhance the growth of DENA-initiated hepatocytes. Indeed, liver sections from rats treated with 2,4-DAT (30 daily doses of 25 mg/kg, i.g.) exhibited an average total number and area of foci of 10.53/cm^2 and 1.22 mm^2/cm^2 vs. 4.46/cm^2 and 0.33 mm^2/cm^2, for their respective controls. By contrast, no effect on the growth of GGT-positive foci was observed when liver sections from rats treated with 2,6-DAT (30 daily doses of 50 mg/kg, i.g.) were scored (5.54 foci per cm^2 and total area of 0.42 mm^2/cm^2). The results indicate that in spite of the structural alert common to the two isomers, 2,4-DAT and 2,6-DAT, only the former appears to significantly affect the carcinogenic process in the liver.

Keywords: 2,4-Diaminotoluene; 2,6-Diaminotoluene; Rat liver; In vivo DNA adducts; Preneoplastic foci

Abbreviations: 2,4-DAT, 2,4-diaminotoluene; 2,6-DAT, 2,6-diaminotoluene; DENA, diethylnitrosamine; PB, phenobarbital; PEI, polyethyleneimine; TLC, thin-layer chromatography; RAL, relative adduct labelling; PH, partial hepatectomy; GGT, γ-glutamyltranspeptidase; 2-AAF, 2-acetylaminofluorene; TD_{50}, tumorigenic dose rate 50.

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1. Introduction

In the chemical class of aromatic amines, 2,4-diaminotoluene (2,4-DAT) and 2,6-diaminotoluene (2,6-DAT) represent a conflicting couple of isomers. Despite showing the same structural alert to DNA reactivity (Ashby and Tennant, 1991), they are different in terms of carcinogenicity, the 2,4-isomer alone being recognized as a potent rodent carcinogen. Under the bioassay conditions of the U.S National Cancer Institute, testing in rats and mice has concluded 2,6-DAT to be non-carcinogenic, while 2,4-DAT has been seen as a transpecies, multiple-site carcinogen, of which the common target, in female mice and in rats of both sexes, is the liver (USDHEW, 1979, 1980). Both chemicals find industrial application as intermediates for the production of various dyes, elastomers and polyurethane foams (USDHEW, 1979, 1980) and, in this last context, their release from polyurethane-encapsulated prostheses used in plastic surgery has been reported (Batich et al., 1989; Chan et al., 1991). An intriguing possibility is that the two chemicals have profoundly different activities in vivo, which could be related to their different carcinogenic potential.

According to the literature, assays in different short-term genotoxicity tests have failed to discriminate satisfactorily between the carcinogenic and non-carcinogenic isomer (George and Westmoreland, 1991), both chemicals showing overall positive results which seem to essentially reflect their common structural alert, i.e. the presence in their molecule of a fragment potentially capable of generating an electrophilic metabolite, as described by Ashby and Tennant (1991). Taking examples for various testing endpoints, both chemicals proved mutagenic in *Salmonella* and in vitro transforming agents (Greene and Friedman, 1980; Furlong et al., 1987; George and Westmoreland, 1991), positive, though weakly, for micronuclei induction in rat bone marrow (George and Westmoreland, 1991), and positive in an assay measuring testicular DNA synthesis reduction in mice (Greene et al., 1981). In addition, although ineffective in V79 cells (Fassina et al., 1985), 2,4-DAT proved mutagenic in L5178Y mouse lymphoma and CHO-AT3-2 cells (Coppinger et al., 1984). In vivo, it was negative for sperm abnormalities in mice (Soares and Lock, 1980; Topham, 1980), but induced sex-linked recessive lethal mutations in *Drosophila* (Blijleven, 1977) and SCEs in mouse bone-marrow cells (Topham, 1980; Parodi et al., 1983). Moreover, 2,6-DAT was also found to induce chromosome aberrations in CHO and CHL cells (Gulati et al., 1989; Bean and Galloway, 1993). On the whole, the effects measured in these tests point out no clear evidence for a different potency between 2,4- and 2,6-DAT.

With specific regard to liver cells as a target, in vitro tests found 2,4-DAT negative for DNA fragmentation (Sina et al., 1983) and positive for unscheduled DNA synthesis both in human and rat hepatocytes, 2,6-DAT showing a positive and a negative result in the same cell types, respectively (Butterworth et al., 1989). Tests for the same genotoxicity endpoints, but using in vivo treatments, seemed a more, perhaps the most, appropriate way for discriminating between 2,4-DAT and 2,6-DAT. In these, 2,4-DAT proved clearly positive for DNA fragmentation (Parodi et al., 1981; Brambilla et al., 1985), while 2,6-DAT gave positive/negative results under different experimental conditions (Allavena et al., 1992). In terms of unscheduled DNA synthesis, discrimination between the two isomers was achieved (Mirsalis et al., 1982), although this was not always sharp (George and Westmoreland, 1991; Allavena et al., 1992). In addition, covalent DNA binding of both isomers (approximately four times greater for 2,4-DAT than for 2,6-DAT) was found in in vitro rat hepatocytes (Furlong et al., 1987). More recently, the 2,4-isomer was shown to induce DNA adducts in various organs of F-344 rats, especially in liver and mammary glands, which are targets for its carcinogenicity (La and Froines, 1992). Moreover, the different carcinogenic behaviour of these potentially genotoxic isomers has been suggested to depend on a difference in a non-genotoxic activity, such as the induction of cell proliferation. In this regard, a clear-cut discrimination was observed in the liver of Fischer rats, in which 2,4-DAT, but not 2,6-DAT, induced a positive, dose-dependent effect after nine daily doses (Cunningham et al., 1991). As the above-mentioned data are, on the whole, rather fragmentary and have

yielded scant information, we decided to run simultaneous tests for genotoxic and non-genotoxic activity on both 2,4-DAT and 2,6-DAT, in the most appropriate target, i.e. rat liver in vivo, with a view to obtaining clearer insights into the differing liver carcinogenicity of these chemicals and to better define their respective activity patterns. The isomers were studied for their ability to induce DNA adducts, as detected by the ^{32}P-postlabelling technique, and to enhance the induction of preneoplastic foci, as detected in diethylnitrosamine (DENA)-initiated hepatocytes.

The aim of our study was to discern whether, using the correct target/metabolism, a classic genotoxicity assay and an assay detecting non-genotoxic activities could, together, correctly reflect the different carcinogenic behaviour of two isomers, which, on the basis of their structural formulae and after assay in the usual tests, appear to have a similar carcinogenic potential.

2. Materials and methods

2,4-DAT (98% pure, CAS No. 95-80-7) and 2,6-DAT (97% pure, CAS No. 823-40-5) were purchased from Aldrich (Steinheim, Germany). Phenobarbital (PB) sodium salt (CAS No. 57-30-7) was from BDH Italiana (Milano, Italy) and DENA (CAS No. 55-18-5) from Sigma (St. Louis, MO). Gamma-^{32}P-ATP (3000 Ci/mmol) was from DuPont (Wilmington, DE), while T4 polynucleotide kinase was from Pharmacia (Uppsala, Sweden). Both polyethyleneimine (PEI)-cellulose sheets, used for thin-layer chromatography (TLC), and C-18 (RP-18 F_{254}s) sheets, used for reversed-phase TLC, were purchased from Merck (Darmstadt, Germany). All other chemicals and biochemicals were of common laboratory use. Male Fischer-344 rats (160–180 g b.w) were purchased form Charles River Italiana (Calco, CO, Italy).

For ^{32}P-postlabelling experiments, livers of five control or treated rats (see legend to Fig. 1) were pooled and DNA isolated (Reddy and Randerath, 1987). DNA adducts were ^{32}P-postlabelled after nuclease P1 digestion of normal nucleotides according to Reddy and Randerath (1986), with minor modifications. Samples of 10 μg DNA were hydrolyzed for 3.5 h at 37°C to 3′-mononucleotides in 15-μl mixtures containing 8 mU of spleen phosphodiesterase and 0.4 U of micrococcal nuclease. DNA hydrolysates were evaporated to dryness and appropriately redissolved. Then, their samples of 8 μg/9 μl were enriched in adducted nucleotides by incubation with 4 μg nuclease P1 for 40 min at 37°C, according to Peluso et al. (1991). Adducted nucleotides were subsequently ^{32}P-labelled at the 5′-termini by incubation, for 40 min at 37°C, with 75 μCi of carrier-free γ-^{32}P-ATP and 5 U of T4 polynucleotide kinase, in 25 μl of bicine-buffered mixtures (pH 9), which were composed as previously described (Peluso et al., 1991). Excess ATP in the reaction mixtures and efficiency of the removal of normal nucleotides were checked by analysing samples of the labelled post-nuclease P1 digests, by one-dimensional PEI-cellulose TLC (Randerath et al., 1988). To purify and separate ^{32}P-postlabelled nucleotide adducts, the labelled mixtures were then applied to C-18 reversed-phase TLC plates and developed in 0.4 M ammonium formate (pH 6.2) according to Randerath et al. (1984). Adducted nucleotides remaining at the origin were transferred onto PEI-cellulose plates by n-propanol/H_2O (1:1 v/v), 1% Nonidet P-40, then separated by two-dimensional TLC (Randerath et al., 1984). Spots were excised and measured for radioactivity by liquid scintillation counting, and DNA adduct levels measured as relative adduct labelling (RAL), i.e. counts/min in adducted nucleotides/counts/min in total nucleotides (Reddy and Randerath, 1986). To calculate counts/min in total nucleotides, samples of DNA hydrolysates were appropriately diluted and reacted in mixtures as described above for ^{32}P-postlabelling of the adducted nucleotides; the obtained ^{32}P-labelled normal nucleotides were then separated by one-dimensional PEI-cellulose TLC and counted for their radioactivity (Geneste et al., 1991).

For the preneoplastic foci test, groups of six rats were initiated at the same time by i.p. injection of 200 mg/kg DENA (Ito et al., 1988). After 2 weeks on basal diet, treated groups were given the test chemicals, 2,4-DAT or 2,6-DAT, in daily oral doses of 25 mg/kg or 50 mg/kg, respectively. The doses are identical to those tested by Cunningham

et al. (1991) for cell proliferation in rat liver, and comparable to the daily dose rates of 250 and 500 ppm tested in long-term bioassays (USDHEW, 1979, 1980). Both 2,4-DAT and 2,6-DAT were administered in 0.01 M HCl by gavage 5 days/week. Rats treated as positive controls were exposed to 500 ppm PB in their drinking water (Ford and Pereira, 1980). In accordance with the methods of Ito et al. (1988), partial hepatectomy (PH) was performed at week 3 in rats of all groups, and the

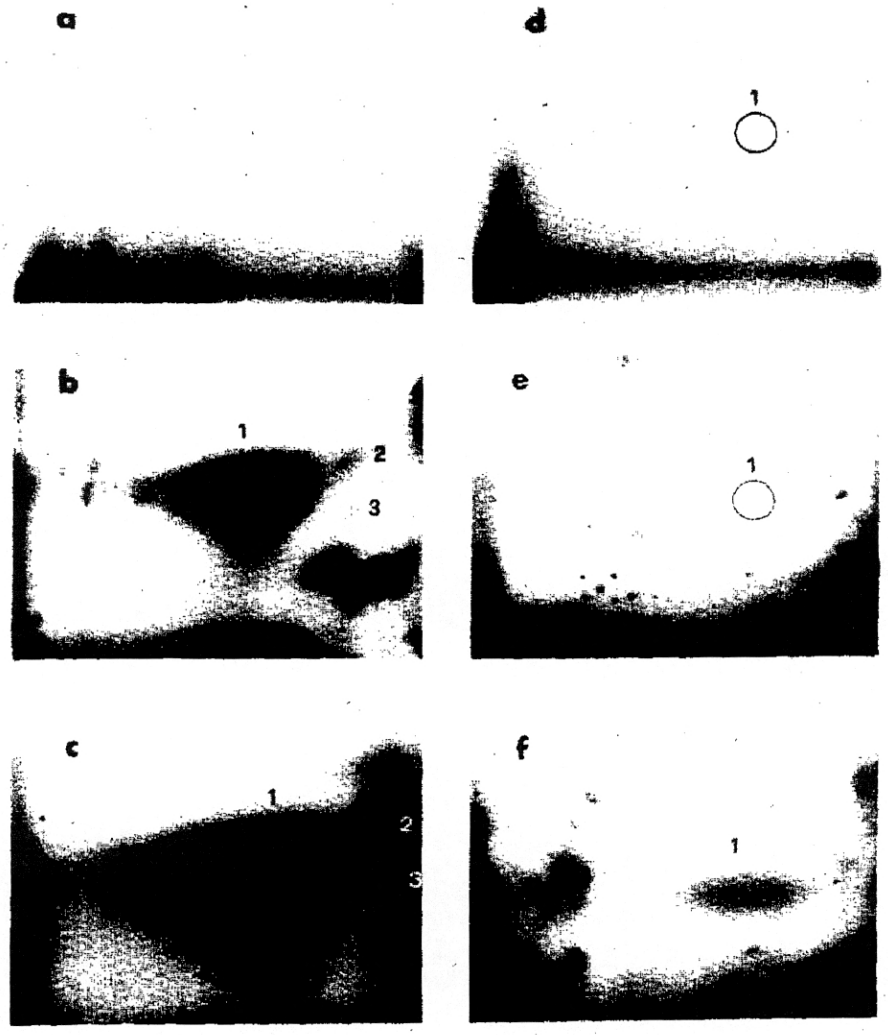

Fig. 1. Autoradiograms of ^{32}P-labelled liver DNA adducts after their separation onto PEI-cellulose TLC plates. For the experiments, F-344 rats were injected i.p. with the test chemicals dissolved in dimethyl sulfoxide and sacrificed 18 h later. Controls received vehicle alone. Panels a–d show autoradiograms of controls (a), and of rats treated with 125 mg/kg 2,4-DAT (b), 250 mg/kg 2,4-DAT (c) and 500 mg/kg 2,6-DAT (d). Panels e, f show long-exposure autoradiograms from rats treated with 250 mg/kg 2,6-DAT and 500 mg/kg 2,6-DAT, respectively. Screen-intensified autoradiography was performed at −80°C, using Kodak XAR-5 films. Exposure time of autoradiograms in panels a–d was 1 h, while that of autoradiograms in panels e, f was 14 h. Spots requiring longer exposure time for detection have been circled.

experiments were terminated at week 8, i.e. after 6-week treatments. Liver sections obtained after sacrifice were fixed in 2-methyl butane which was cooled in liquid nitrogen and stored at −80°C. In liver slices, foci positive for γ-glutamyltranspeptidase (GGT) of ≥0.2 mm mean diameter were scored, after staining according to Rutenburg et al. (1969). Four sections, one each from the right posterior and caudate lobes and two from the right anterior lobe, were used for scoring.

3. Results

As shown in Fig. 1 both 2,4-DAT and 2,6-DAT induced DNA adducts in the liver of male Fischer rats, but to very different extents. While no effect was observed in controls, the autoradiograms of 2,4-DAT displayed one major and two faint spots after 1-h exposure at −80°C (panels a–c). Considering that the irregular size of the major spot may represent more than one altered nucleotide, this result indicates the induction of at least three different types of DNA adducts. In each chromatogram, ^{32}P-radioactivity counts of the adduct spots were pooled. This was done on the grounds that our goal was not to characterize DNA adducts, but to compare the isomers for their actual genotoxicity in the liver target. In this way we found a dose-dependent effect with estimated RAL values, i.e. adduct frequencies in normal nucleotides, of $10.8 \pm 2.4 \times 10^{-6}$ and $18.6 \pm 2.3 \times 10^{-6}$ after test doses of 125 and 250 mg/kg, respectively. Values are means ± S.E. of three independent evaluations. In Fig. 1 (panel d), even at the highly toxic dose of 500 mg/kg (2/5 treated rats survived), no adduct spots were observed for 2,6-DAT after 1-h autoradiography of chromatograms. A longer exposure-time of 14 h allowed us to detect one faint 2,6-DAT adduct spot (Fig. 1, panels e, f), chromatographically similar to the major spot produced by 2,4-DAT, though qualitative identification of DNA adducts was not our intention in the present work. The RAL values obtained for 2,6-DAT were equal to $0.29 \pm 0.06 \times 10^{-8}$ and $5.0 \pm 1.5 \times 10^{-8}$ for the test doses of 250 and 500 mg/kg, respectively. Moreover, a similar pattern of results had been obtained in previous experiments (data not shown) on the liver of female CD-1 mice 18 h after single 2,4-DAT or 2,6-DAT administrations. In those experiments, 1-h autoradiograms yielded negative results after 240 mg/kg 2,6-DAT; by contrast, after 60 mg/kg and 120 mg/kg 2,4-DAT, adduct spots with RAL values of 17.7×10^{-6} and 22.2×10^{-6}, respectively, were observed.

Table 1 reports the changes in body and liver weights, and in preneoplastic foci induction, observed in rats treated to test 2,4-DAT- and 2,6-DAT-promoting activity. For comparison, data obtained on rats treated with PB, a widely used non-genotoxic liver tumor promoter (Schulte-Hermann, 1985), are also reported. As shown, after DENA initiation, 2,4-DAT and 2,6-DAT treatments induced a similar decrease in body-weight gain, while only 2,4-DAT produced a significant increase ($P < 0.005$) in liver/body weight

Table 1
Effect of 2,4- and 2,6-diaminotoluene on body and liver weights, and on liver preneoplastic foci induction in male Fischer-344 rats

Group	Treatment[a]	Body weight (g)	Liver weight (g)	Foci number (No/cm^2)	Foci total area (mm^2/cm^2)
1	DENA + PH	269 ± 5	7.62 ± 0.29	4.46 ± 1.12	0.33 ± 0.08
2	DENA + PH + 2,4-DAT	249 ± 3	9.96 ± 0.41	10.53 ± 1.65*	1.22 ± 0.27*
3	DENA + PH + 2,6-DAT	245 ± 6	7.01 ± 0.28	5.54 ± 1.04	0.42 ± 0.08
4	DENA + PH + PB	291 ± 5	9.95 ± 0.38	15.08 ± 2.35*	1.51 ± 0.30*

Values reported are means ± S.E. of six rats. Foci number and total area are expressed per unit area of liver slide.

[a]Treatments were: DENA, diethylnitrosamine at the single i.p. dose of 200 mg/kg; PH, 2/3 partial hepatectomy; 2,4-DAT, 2,4-diaminotoluene 25 mg/kg i.g. 5 days/week for 6 weeks; 2,6-DAT, 2,6-diaminotoluene 50 mg/kg i.g. 5 days/week for 6 weeks; PB, phenobarbital (sodium salt) 500 ppm in drinking water for 6 weeks. Details in Materials and methods, Section 2.

*Significantly different from Group 1 at $P < 0.05$ (Student's t-test).

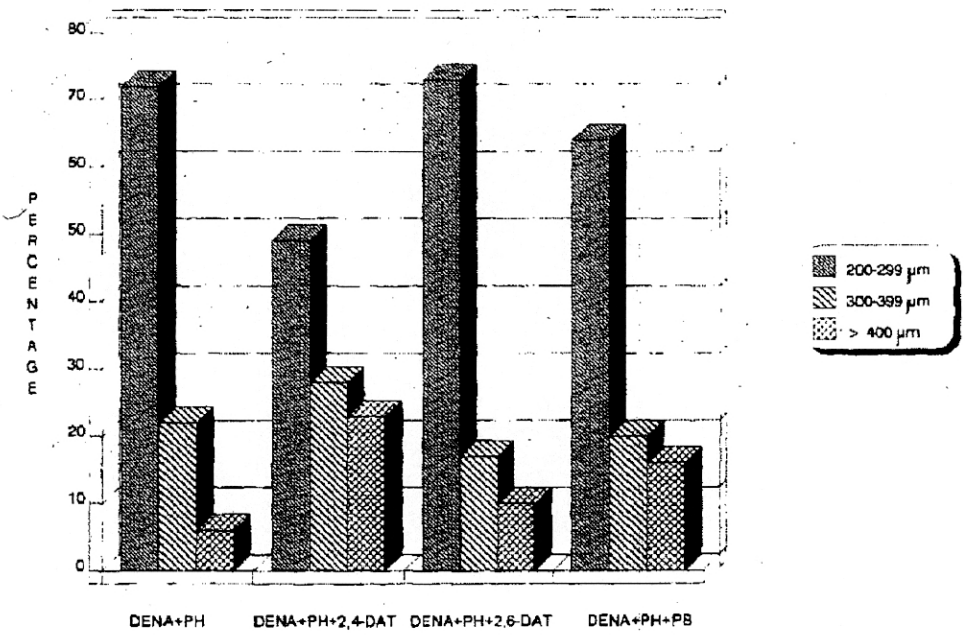

Fig. 2. Diameter size distribution of liver preneoplastic foci in DENA-initiated F-344 rats. Abbreviations and treatment schedules are described in footnote to Table 1, and in Materials and methods, Section 2.

ratio (0.04), which was greater than that induced by PB (~0.034). This is in keeping with the finding of Cunningham et al. (1991), who observed a mitogenic effect for 2,4-DAT, but not for 2,6-DAT, in rat liver. As Table 1 also shows, of the two isomers only 2,4-DAT induced significant increases in preneoplastic foci in our assay, both in terms of number and total area. The overall effect of 2,4-DAT was less pronounced than that of PB. However, as can be inferred from the data listed in Table 1, the mean diameter of foci was larger in 2,4-DAT-treated than in PB-treated samples (384 μm vs. 357 μm). The stimulatory effect of 2,4-DAT on foci size has been detailed in Fig. 2, which reports the diameter size distributions of the scored foci, following treatment with 2,4-DAT, 2,6-DAT and phenobarbital, in comparison with controls.

4. Discussion

After 2,4-DAT treatment with 125 and 250 mg/kg (1023 and 2046 μmol/kg), we found total RAL values of 10.8 and 18.6 × 10^{-6}, respectively. These are in keeping with the values, ~3.5 and ~5 times lower, respectively, obtained by La and Froines (1992) after 1000 and 2046 μmol/kg under similar treatment conditions (see Table 1 and Fig. 2 of their paper). The adduct-spot pattern we obtained is also in keeping with that seen by these authors; minor discrepancies might be due to the use of different batches of C-18 reversed-phase TLC plates, or to differences in digestion time or enzyme concentrations (Savela et al., 1989). On these bases, we found a much weaker effect for 2,6-DAT, which, after 250 mg/kg treatment, gave a RAL value of 0.29 × 10^{-8} (i.e. about 6,500 times lower than that of the 2,4-isomer at equimolar dose) and, after the highly lethal treatment of 500 mg/kg, a RAL of 5 × 10^{-8}. Moreover, the non-linear dose-dependent effect we observed for 2,4-DAT may be related to a flattening out of the dose-response curve, stemming from activating enzyme saturation or dose-dependent changes in

DNA repair processes (La and Froines, 1992). On the contrary, a different pattern of dose-dependent effects was seen for 2,6-DAT, which could imply differences in metabolism between the isomers or, more simply, a non-homogeneous response of rats treated with the highest 2,6-DAT dose. Qualitative and/or quantitative differences in pharmacokinetics (probably less important), or enzymatic activation/detoxification balance (probably more important), between the isomers could account for the large difference we observed. In this view, a slower excretion and a higher liver uptake of 2,4-DAT, in respect to 2,6-DAT, is suggested by the data obtained in rats by Grantham et al. (1980) and Cunningham et al. (1989), though after different treatment types. This suggestion could be supported by the lower, statistically significant difference in DNA adduct induction observed in vitro by Furlong et al. (1987) after exposure of rat hepatocytes to the two isomers at 100 μM concentration. In those experiments in vitro, both chemicals were found to bind DNA after metabolic activation implying both cytochrome P-450 and sulfation (Furlong et al. 1987). In rats, after analysis of the urinary metabolites, both chemicals were additionally found to be oxidized and/or acetylated (Grantham et al., 1980; Cunningham et al., 1989). Stereospecific enzymatic reactions could, however, produce qualitative differences, or different rates of DNA-reactive and/or inactive metabolites, responsible for a different DNA adduct yield.

The genotoxic potential of 2,4- and 2,6-DAT is determined by the presence, in their molecule, of fragments structurally alerting for DNA reactivity. A structural alerting fragment can be defined as a molecular fragment, generally but not necessarily, made of contiguous atoms which may interact with a specific receptor site of an effector molecule, in this case an enzyme performing metabolic activation of the substrate with the final formation of an electrophilic metabolite. According to Ashby and Tennant (1991), an aminogroup linked to a benzene ring can be considered in principle as a structural alert for genotoxicity, provided the aminogroup metabolic activation is not hindered by adjacent fragments. From this standpoint, both 2,4- and 2,6-DAT share the same genotoxic potential. Finer characterisations of the putative structural alert are nonetheless possible; for instance, the presence in the benzene ring of two aminogroups separated by a single nonsubstituted carbon atom could ensure a higher affinity to the activating enzyme(s) than the presence of a single aminogroup. In this situation, despite both isomers sharing common molecular fragments, they are different for some fragment structures. We advance the hypothesis that one of the fragment structures present in 2,4-, but not in 2,6-DAT, could be the true discriminating structural alert for a different DNA reactivity, as observed in our experiments. To clarify this at the level of structure-activity relationships it may be useful to study, for DNA adduct formation in vivo, additional congeneric molecules of the diaminotoluene family.

To put our results in perspective, using literature and our present data, one can compare the induction of DNA adducts in rat liver by 2,4-DAT, 2,6-DAT and 2-acetylaminofluorene (2-AAF), a representative genotoxic compound (IPCS, 1988), which also belongs to the aminoaromatic chemical class (Ashby and Tennant, 1991). Moreover, the carcinogenic potential of these chemicals in liver, defined as tumorigenic dose rate 50 (TD_{50}) according to Peto et al. (1984), and expressed here in μmol/kg/day, can be also compared. Despite the insufficient data to consider 2,6-DAT as a rodent carcinogen, as defined by the U.S. National Cancer Institute guidelines (USDHEW, 1980), the carcinogenic potential displayed by this chemical was utilized for our computations. This was done considering both the significant, dose-related trend of tumor induction which was specifically observed in mouse and rat liver after 2,6-DAT administration (USDHEW, 1980) and the availability of a significant TD_{50} value for this chemical, as reported in the database by Gold et al. (1986). On these grounds, at the dose of ~170 μmol/kg, 2-AAF induces 2–100 adducts per million nucleotides (Randerath et al., 1985) and, in our experiments, at the mean doses of ~1500 and ~3000 μmol/kg, respectively, 2,4-DAT and 2,6-DAT induce 10–20 and 0.003–0.05 adducts per million nucleotides, respectively. It should be remembered that the extent of DNA binding, nor-

malized on a molar basis for the administered dose, has been demonstrated by Lutz (1979) to have a good quantitative correlation with the hepatocarcinogenic potency of disparate chemicals and, moreover, that the ^{32}P-postlabelling technique is able to detect weakly genotoxic compounds, which either are not carcinogenic or whose carcinogenicity is not readily shown in animal bioassays (Randerath et al., 1985). In keeping with the pattern for DNA adduct induction, TD_{50} values extrapolated from the literature (Gold et al., 1984, 1986, 1990) are approximately 2.7, 66 and 600 μmol/kg/day for 2-AAF, 2,4-DAT and 2,6-DAT, respectively. In our extrapolation, we chose the lowest value, corresponding to the highest potency, when more than one positive result was available. Obviously, because of non-homogeneity in experimental protocols, the comparison can only be taken as indicative. It should be added that qualitative differences between DNA adducts induced by 2,4-DAT and 2,6-DAT could lead to different tumor yields. Also, adduct levels observed after single dose treatments may not accurately reflect those from chronic studies, as indicated by La and Froines (1992). Even so, our comparison seems to indicate that the assays we used are, paradoxically, too sensitive to the different carcinogenic potential of 2,4- and 2,6-DAT.

Concerning the non-genotoxic-promoting activity of the two isomers on DENA-initiated hepatocytes, 2,6-DAT revealed negligible, not significant effects, while 2,4-DAT induced statistically significant increases in preneoplastic foci, as phenotypically evaluated by GGT-staining. Quantitatively, this effect was less marked than that induced by PB, a representative liver tumor promoter endowed with carcinogenic activity (Gold et al., 1984, 1986, 1990). Following 2,4-DAT treatment, the increase in the total area of foci was greater than the increase in their number. This suggests that the chemical increases the proliferation of initiated hepatocytes and, therefore, also the number of preneoplastic foci detectable at the observation time, in our experimental model. This also seems evident when the diameter size distributions of foci from control and 2,4-DAT treated rats are compared (Fig. 2). Moreover, the positive effect observed for 2,4-DAT is in keeping with the results by Cunningham et al. (1991) who, in conditions similar to ours, found that 2,4-DAT, but not 2,6-DAT, induced significant dose-dependent hepatocyte proliferation in non-initiated rat liver in vivo. It should be added that no data are available to determine to what extent 2,4-DAT genotoxicity contributes to cell-proliferating activity, either by a direct stimulation of initiated cells or by an indirect effect, i.e. DNA adducts followed by cell toxicity and death, in turn followed by cell regeneration, or moreover, to what extent stimulation of cell proliferation contributes to the carcinogenicity-promoting activity of 2,4-DAT. Nevertheless, our results help to define the activity patterns of 2,4-DAT and 2,6-DAT and indicate that, in spite of their common structural bent for DNA reactivity, only the former isomer appears to significantly affect the carcinogenic process in the liver, as evaluated by DNA adduct and GGT-positive preneoplastic foci induction.

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References

Allavena, A., Martelli, A., Robbiano, L. and Brambilla, G. (1992) Evaluation in a battery of in vivo assays of four in vitro genotoxins proved to be noncarcinogens in rodents. Teratogen. Carcinogen. Mutagen. 12, 31–41.

Ashby, J. and Tennant, R.W. (1991) Definitive relationships among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the U.S. NTP Mutat. Res. 257, 229–306.

Batich, C., Williams, J. and King, R. (1989) Toxic hydrolysis product from a biodegradable foam implant. J. Biomed. Mater. Res. 23, 311–319.

Bean, C.L. and Galloway, S.M. (1993) Evaluation of the need

for a late harvest time in the assay for chromosome aberrations in Chinese hamster ovary cells. Mutat. Res. 292, 3–16.

Blijleven, W.G.H. (1977) Mutagenicity of four hair dyes in *Drosophila melanogaster*. Mutat. Res. 48, 181–186.

Brambilla, G., Carlo, P., Finollo, R. and Ledda, A. (1985) Viscometric detection of liver DNA fragmentation in rats treated with ten aromatic amines. Discrepancies with results provided by the alkaline elution technique. Carcinogenesis 6, 1285–1288.

Butterworth, B.E., Smith-Oliver, T., Earle, L., Loury, D.J., White, R.D., Doolittle, D.J., Working, P.K., Cattley, R.C., Jirtle, R., Michalopoulos, G. and Strom, S. (1989) Use of primary cultures of human hepatocytes in toxicology studies. Cancer Res. 49, 1075–1084.

Chan, S.C., Birdsell, D.C. and Gradeen, C.Y. (1991) Urinary excretion of free toluenediamines in a patient with polyurethane-covered breast implants. Clin. Chem. 37, 2143–2145.

Coppinger, W.J., Brennan, S.A., Carver, J.H. and Thompson, E.D. (1984) Locus specificity of mutagenicity of 2,4-diaminotoluene in both L5178Y mouse lymphoma and AT3-2 Chinese hamster ovary cells. Mutat. Res. 135, 115–123.

Cunningham, M.L., Burka L.T. and Matthews, H.B. (1989) Metabolism, disposition, and mutagenicity of 2,6-diaminotoluene, a mutagenic noncarcinogen. Drug Metab. Dispos. 17, 612–617.

Cunningham, M.L., Foley, J., Maronpot, R.R. and Matthews, H.B. (1991) Correlation of hepatocellular proliferation with hepatocarcinogenicity induced by the mutagenic noncarcinogen:carcinogen pair — 2,6- and 2,4-diaminotoluene. Toxicol. Appl. Pharmacol. 107, 562–567.

Fassina, G., Abbondandolo, A. and Parodi, S. (1985) Mutation induction by aromatic amines in V79 Chinese hamster cells. Mutat. Res. 147, 292–293.

Ford, J.O. and Pereira, M.A. (1980) Short-term in vivo initiation/promotion bioassay for hepatocarcinogens. J. Toxicol. Environ. Health 4, 39–46.

Furlong, B.B., Weaver, R.P. and Goldstein, J.A. (1987) Covalent binding to DNA and mutagenicity of 2,4-diaminotoluene metabolites produced by isolated hepatocytes and 9000 × *g* supernatant from Fischer-344 rats. Carcinogenesis 8, 247–251.

Geneste, O., Camus, A.M., Castegnaro, M., Petruzzelli, S., Macchiarini, P., Angeletti, C.A., Giuntini, C. and Bartsch, H. (1991) Comparison of pulmonary DNA adduct levels, measured by ^{32}P-postlabelling and aryl hydrocarbon hydroxylase activity in lung parenchyma of smokers and ex-smokers. Carcinogenesis 12, 1301–1305.

George, E. and Westmoreland, C. (1991) Evaluation of the in vivo genotoxicity of the structural analogues 2,6-diaminotoluene and 2,4-diaminotoluene using the rat micronucleus test and rat liver UDS assay. Carcinogenesis 12, 2233–2237.

Gold, L.S., Sawyer, C.B., Magaw, R., Backman, G.M., de Veciana, M., Levinson, R., Hooper, N.K., Havender, W.R., Bernstein, L., Peto, R., Pike, M.C. and Ames, B.N. (1984) A carcinogenic potency database of the standardized results of animal bioassays. Environ. Health Perspect. 58, 9–319.

Gold, L.S., de Veciana, M., Backman, G.M., Magaw, R., Lopipero, P., Smith, M., Blumenthal, M., Levinson, R., Bernstein, L. and Ames, B.N. (1986) Chronological supplement to the carcinogenic potency database: standardized results of animal bioassays published through December 1982. Environ. Health Perspect. 67, 161–200.

Gold, L.S., Slone, T.H., Backman, G.M., Eisenberg, S., Da Costa, M., Wong, M., Manley, N.B., Rohrbach, L. and Ames, B.N. (1990) Third chronological supplement to the carcinogenic potency database: standardized results of animal bioassays published through December 1986 and by the National Toxicology Program through June 1987. Environ. Health Perspect. 84, 215–285.

Grantham, P.H., Mohan, L., Benjamin, T., Roller, P.P., Miller, J.R. and Weisburger E.K. (1980) Comparison of the metabolism of 2,4-toluenediamine in rats and mice. J. Environ. Pathol. Toxicol. 3, 149–166.

Greene, E.J. and Friedman, M.A. (1980) In vitro cell transformation screening of four toluenediamine isomers. Mutat. Res. 79, 363–375.

Greene, E.J., Salerno, A.J. and Friedman, M.A. (1981) Effect of four toluenediamine isomers on murine testicular DNA synthesis. Mutat. Res. 91, 75–79.

Gulati, D.K., Witt, K., Anderson, B., Zeiger, E. and Shelby, M.D. (1989) Chromosome aberration and sister chromatid exchange tests in Chinese hamster ovary cells in vitro. III. Results with 27 chemicals. Environ. Mol. Mutagen. 13, 133–193.

IPCS (1988) Evalutaion of Short-Term Tests for Carcinogens. In: J. Ashby, F.J. de Serres, M.D. Shelby, B.H. Margolin, M. Ishidate, Jr. and G.C. Becking (Eds), International Programme on Chemical Safety, Cambridge University Press, Cambridge, Vols. I and II.

Ito, N., Tsuda, H., Tatematsu, M., Inoue, I., Tagawa, Y., Aoki, T., Uwagawa, S., Kagawa, M., Ogiso, T., Masui, T., Imaida, K., Fukushima, S. and Asamoto, M. (1988) Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione *S*-transferase placental form positive foci in rats — an approach for a new medium-term bioassay system. Carcinogenesis 9, 387–394.

La, D.K. and Froines, J.R. (1992) ^{32}P-Postlabelling analysis of DNA adducts from Fischer-344 rats administered 2,4-diaminotoluene. Chem.-Biol. Interact. 83, 121–134.

Lutz, W.K. (1979) In vivo covalent binding of organic chemicals to DNA as a quantitative indicator in the process of chemical carcinogenesis. Mutat. Res. 65, 289–356.

Mirsalis, J.C., Tyson, C.K. and Butterworth, B.E. (1982) Detection of genotoxic carcinogens in the in vivo-in vitro hepatocyte DNA repair assay. Environ. Mutagen. 4, 553–562.

Parodi, S., Taningher, M., Russo, P., Pala, M., Tamaro, M. and Monti-Bragadin, C. (1981) DNA-damaging activity in vivo and bacterial mutagenicity of sixteen aromatic amines and azo-derivatives, as related quantitatively to their carcinogenicity. Carcinogenesis 2, 1317–1326.

Parodi, S., Zunino, A., Ottaggio, L., De Ferrari, M. and Santi, L. (1983) Lack of correlation between the capability of inducing sister chromatid exchanges in vivo and carcinogenic potency, for 16 aromatic amines and azo-derivatives. Mutat. Res. 74, 379–387.

Peluso, M., Castegnaro, M., Malaveille, C., Friesen, M., Garren, L., Hautefeuille, A., Vineis, P., Kadlubar, F. and Bartsch, H. (1991) ^{32}P-Postlabelling analysis of urinary mutagens from smokers of black tobacco implicates 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) as a major DNA-damaging agent. Carcinogenesis 12, 713–717.

Peto, R., Pike, M.C., Bernstein, L., Gold, L.S. and Ames, B.N. (1984) The TD_{50}: a proposed general convention for the numerical description of the carcinogenic potency of chemicals in chronic-exposure animal experiments. Environ. Health Perspect. 58, 1–8.

Randerath, K., Haglund, R.E., Phillips, D.H. and Reddy, M.V. (1984) ^{32}P-Postlabeling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. Carcinogenesis 5, 1613–1622.

Randerath, K., Randerath, E., Agrawal, H.P., Gupta, R.C., Schurdak, M.E. and Reddy, M.V. (1985) Postlabeling methods for carcinogen-DNA adduct analysis. Environ. Health Perspect. 62, 57–65.

Randerath, E., Mittal, D. and Randerath, K. (1988) Tissue distribution of covalent DNA damage in mice treated dermally with cigarette 'tar': preference for lung and heart DNA. Carcinogenesis 9, 75–80.

Reddy, M.V. and Randerath, K. (1986) Nuclease P_1-mediated enhancement of sensitivity of ^{32}P-postlabelling test for structurally diverse DNA adducts. Carcinogenesis 7, 1543–1551.

Reddy, M.V. and Randerath, K. (1987) ^{32}P-analysis of DNA adducts in somatic and reproductive tissues of rats treated with the anticancer antibiotic, mitomycin C. Mutat. Res. 179, 75–88.

Rutenburg, A.M., Kim, H., Fishbein, J.W., Hanker, S.S., Wasserkrug, H.L. and Seligman, A.M. (1969) Histochemical and ultrastructural demonstration of gamma-glutamyltranspeptidase activity. J. Histochem. Cytochem. 17, 517–526.

Savela, K., Hemminki, K., Hewer, A., Phillips, D.H., Putman, K.L. and Randerath, K. (1989) Interlaboratory comparison of the ^{32}P-postlabelling assay for aromatic DNA adducts in white blood cells of iron foundry workers. Mutat. Res. 224, 485–492.

Schulte-Hermann, R. (1985) Tumor promotion in the liver. Arch. Toxicol. 57, 147–158.

Sina, J.F., Bean, C.L., Dysart, G.R., Taylor, V.I. and Bradley, M.O. (1983) Evaluation of the alkaline elution/rat hepatocyte assay as a predictor of carcinogenic/mutagenic potential. Mutat. Res. 113, 357–391.

Soares, E.R. and Lock, L.F. (1980) Lack of an indication of mutagenic effects of dinitrotoluenes and diaminotoluenes in mice. Environ. Mutagen. 2, 111–124.

Topham, L.C. (1980) Do induced sperm-head abnormalities in mice specifically identify mammalian mutagens rather than carcinogens? Mutat. Res. 74, 379–387.

USDHEW (1979) Bioassay of 2,4-diaminotoluene for possible carcinogenicity. U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, NCI Carcinogen. Tech. Rep. Ser. No. 162.

USDHEW (1980) Bioassay of 2,6-toluenediamine hydrochloride for possible carcinogenicity. U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, NCI Carcinogen. Tech. Rep. Ser. No. 200.

EXHIBIT J

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Mutagens that are not carcinogens: faulty theory or faulty tests?

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Abstract

In the National Toxicology Program database of 172 chemicals that were judged non-carcinogenic or equivocal in 2 year rodent studies in both sexes of rats and mice, there are 38 chemicals that were mutagenic in *Salmonella*. All but two of the chemicals had structural alerts for mutagenicity. The largest proportion of the mutagenic non-carcinogens were benzeneamines and substituted benzeneamines. In all, 12 of the mutagenic non-carcinogens had mutagenic carcinogen analogues, and for two chemicals, the carcinogenic analogues were not mutagenic. Non-carcinogens that were mutagenic in *Salmonella* also tended to be mutagenic and clastogenic in mammalian in vitro tests. The mutagenic responses are discussed and explanations offered for the mutagenicity and lack of carcinogenic activity of these chemicals. Published by Elsevier Science B.V.

Keywords: Salmonella; Ames test; Mutagenicity; Carcinogenicity; Non-carcinogen; False-positive

1. Introduction

The dominant paradigm in genetic toxicology is that the ability of a chemical to cause mutations presages its ability to cause cancer. The *Salmonella* mutation (Ames) test has been the most widely used test to identify mutagens among pure substances, in mixtures, and environmental samples. This test uses strains of bacteria containing mutations in the histidine operon which render them unable to grow in the absence of histidine (histidine-dependent), and also contain a number of mutations to make the cells more responsive to xenobiotic mutagens [1]. In the presence of mutagens, one or more of these strains will mutate to wild type (histidine independent) and be identified by their ability to grow and form colonies in the absence of histidine.

Numerous studies have shown that the proportion of carcinogens identified as mutagens by the *Salmonella* test ranges from approximately 50–90% [2–6], depending on the structures of the chemicals being tested and the criteria for their selection, and a positive response in *Salmonella* has a predictivity for rodent carcinogenicity of 77–98% [2–6]. The converse of this positive predictivity is that 2–23% of *Salmonella* mutagens are not carcinogens. In a recent report [6], 33 (21%) of 158 non-carcinogens were *Salmonella* mutagens. This does not fit the paradigm and suggests a number of possibilities.

The *Salmonella* mutagenicity test is most often used as a screen for potential carcinogens and germ cell mutagens, rather than as a definitive test. As such, it has been widely required or recommended by regulatory authorities for pesticides, food additives, and industrial chemicals as well as for monitoring environmental samples. Although the test is used primarily as a screen, a positive result often causes the substance to be branded as a presumptive carcinogen and in many

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situations, the definitive carcinogenicity studies are not performed and the chemical is put aside and not developed further. Chemicals that are not mutagenic in *Salmonella* are not afforded the same presumption of non-carcinogenicity because of the relatively high (approx. 50%) [6] proportion of rodent carcinogens that are not mutagenic, and because the social costs of releasing a cryptic carcinogen for industrial or consumer use may be too high. Depending on the potential uses of the chemical, it is often considered prudent to confirm presumptive non-carcinogens, but to be willing to discard a mutagenic chemical that may not be carcinogenic, or label the chemical as a potential carcinogen in the absence of further testing. In some cases, the costs of disproving the presumption of carcinogenicity may not be justified if there are acceptable non-mutagenic chemicals available.

There have been a number of discussions in the literature regarding the mechanisms and relevance of mutagenic non-carcinogens [7–9], whether this class of chemicals is consistent with the general theories of carcinogenic mechanisms and whether they negate the usefulness of in vitro genetic testing programs. This study identifies the mutagenic non-carcinogens found among the chemicals tested by the US National Toxicology Program (NTP), relates them to structurally analogous carcinogens, and addresses the possible reasons for their mutagenicity in *Salmonella* and their non-carcinogenicity in rodents.

Table 1
Salmonella mutagens judged to be non-carcinogens in both sexes of rats and mice[a]

Chemical	CASRN	SAL	ABS	SCE	MLA	TR#
Calcium cyanamide	156-62-7	+w	−	−	−	163
4′-(Chloroacetyl)acetanilide	140-49-8	+	+	+		177
2-Chloroethanol	107-07-3	+	+	+	+	275
2-Chloromethylpyridine HCl	6959-47-3	+	+	+	+	178
2-Chloro-*p*-phenylenediamine SO_4	61702-44-1	+	+	+	+	113
1-Chloro-2-propanol (technical)	127-00-4	+	+	+		477
3-Chloro-*p*-toluidine	95-74-9	+w	?	−	?	145
Dimethoate	60-51-5	+	?	+		4
2,4-Dimethoxyaniline HCl	54150-69-5	+	+	+	+	171
Dioxathion	78-34-2	+	−	+	−	125
Formulated fenaminosulf	140-56-7	+	−	+	+	101
Glutaraldehyde	111-30-8	+	?	+	+	490
HC Blue 2	33229-34-4	+	−	+	+	293
8-Hydroxyquinoline	148-24-3	+	+w	+	+	276
Iodoform	75-47-8	+	−	+w		66
Lead dimethyldithiocarbamate	19010-66-3	+	+/−[b]	−		151
Methyl parathion	298-00-0	+	−	+		157
N-(1-Naphthyl)ethylenediamine 2HCl	1465-25-4	+	+	+	−	168
4-Nitroanthranilic acid	619-17-0	+	+	+	+	109
1-Nitronaphthalene	86-57-7	+	+w	−		64
4-Nitro-*o*-phenylenediamine	99-56-9	+	−	+	+	180
p-Phenylenediamine 2HCl	624-18-0	+	+	+	+	174
Photodieldrin	13366-73-9	+	−	+		17
Propylene	115-07-1	+			−	272
2,5-Toluenediamine SO_4	6369-59-1	+	+	+	+	126
2,6-Toluenediamine 2HCl	15481-70-6	+	+	+	+	200

[a] SAL: *Salmonella* mutagenicity test; ABS: chromosome aberrations in CHO cells; SCE: sister-chromatid exchanges in CHO cells; MLA: mutations in mouse lymphoma L5178Y cells; TR#: NCI/NTP (carcinogenicity) Technical Report number. The study abstracts for all Technical Report (TR) numbers can be found in [10] and/or [11]; +: positive response; +w: weakly positive response; −: negative response; ?: equivocal response; blank: not tested.

[b] Conflicting results in two independent tests

Table 2
Salmonella mutagens judged to be equivocal carcinogens in rats and mice[a]

Chemical	CASRN	MR	FR	MM	FM	SAL	ABS	SCE	MLA	TR#
Allyl chloride	107-05-1	N	N	E	E	+				73
p-Anisidine (HCl)[b]	20265-97-8	E	N	N	N	+	+	+	+	116
Azinphos methyl	86-50-0	E	N	N	N	+w	+	+		69
1,2,3-Benzotriazole	95-14-7	E	E	N	E	+w	+	+		88
Di(*p*-ethylphenyl) dichloroethane (*p,p'*-Ethyl DDD)	72-56-0	N	N	N	E	+	−	+w		156
Dimethoxane	828-00-2	N	N	E	N	+	+	+		354
Emodin	518-82-1	N	E	E	N	+	+			493
HC Yellow 4	59820-43-8	E	N	N	N	+	−	+		419
3-Nitropropionic acid	504-88-1	E	N	N	N	+	+w	+	+	52
Phosphamidon	13171-21-6	E	E	N	N	+				16
C.I. Pigment red 23	6471-49-4	E	N	N	N	+	−	+		411
Proflavin HCl, 1/2 H_2O	952-23-8	E	N	E	E	+				5

[a] MR: male rat; FR: female rat; MM: male mouse; FM: female mouse; N: not carcinogenic; E: equivocal carcinogen; SAL: *Salmonella* mutagenicity test; ABS: chromosome aberrations in CHO cells; SCE: sister-chromatid exchanges in CHO cells; MLA: mutations in mouse lymphoma L5178Y cells; TR#: NCI/NTP (carcinogenicity) Technical Report number. The study abstracts for all Technical Report (TR) numbers can be found in [10] and/or [11]; +: positive response; +w: weakly positive response; −: negative response; blank: not tested.

[b] Genetic toxicity testing was performed using the free amine (SAL, ABS, SCE) and the HCl (SAL, MLA); the carcinogenicity study was performed using the HCl salt.

2. Databases used

Between 1971 and the present, the US National Cancer Institute (NCI), and subsequently, the NTP, tested more than 475 substances for carcinogenicity in rats and/or mice, and have presented the results in Technical Reports (TR) [10,11]. Of these chemicals, 430 have been tested by the NTP for mutagenicity in *Salmonella* ([12]; unpublished). There were 172 chemicals adequately tested for carcinogenicity in both sexes of rats and mice and judged non-carcinogenic or equivocal. Of these, 38 were judged to be mutagenic in *Salmonella* (Tables 1 and 2).

The *Salmonella* mutagenicity test [13], and the rodent carcinogenicity test protocols [14,15] are described elsewhere. The *Salmonella* test uses tester strains containing specific histidine mutations, without exogenous metabolic activation by liver homogenate (S9) from Aroclor-induced rats and Syrian hamsters. Chemicals are not judged non-mutagenic unless they have been tested in at least four *Salmonella* tester strains (TA98, TA100, TA1535, and TA97 or TA1537). A positive response in any single strain either with or without metabolic activation is sufficient for designating a chemical as a mutagen. Other tester strains are added depending on the responses seen in the initial tests, or on the structure of the test chemical or its anticipated metabolites.

A chemical is not considered to be a non-carcinogen unless it has been chronically administered to male and female rats and mice for up to 2 year. The highest dose tested is usually an maximum tolerated dose/minimally toxic dose (MTD) plus one or two decreasing doses, and a vehicle control. Chemicals may be administered to the animals in feed or water, by inhalation, gavage, or skin painting. A positive response in any one of the sex-species combinations is sufficient for designating a chemical as a carcinogen. An equivocal response in any one of the sex-species combinations, and non-carcinogenic responses in the other three, is sufficient for designating a chemical as "equivocal". Chemicals judged to be equivocal carcinogens based on the responses of one or more sex/species combinations are treated as non-carcinogenic for the purpose of this evaluation.

The carcinogenicity test protocol and data evaluation procedures have evolved since the inception of the testing program, with the greatest changes appearing in the late 1980s. As a rule, the earlier protocols were less sensitive than those initiated for tests commencing around 1980, i.e. some of the protocols used fewer animals, shorter exposure times, or doses that

may not have reached the MTD, and fewer tissues were examined for tumors. In the evaluations reported here, the equivocal carcinogens and non-carcinogens are limited to those adequately tested in both sexes of rats and mice. Chemicals reported as equivocal or non-carcinogenic based solely on testing in a single rodent species have not been included here. Carcinogenicity test results are reported in detail in the published NCI and NTP TR.

3. Results

The mutagenic non-carcinogens are listed in Table 1. The mutagenic, equivocal carcinogens are in Table 2. The carcinogenicity Technical Report summaries can be found in [10,11]. There were 120 of the 172 chemicals tested for carcinogenicity in rats and mice that were judged to be non-carcinogenic, of these 26 (22%) were mutagenic in *Salmonella* (Table 1). Of the 52 chemicals judged equivocal in carcinogenicity tests, 12 (23%) were mutagenic in *Salmonella* (Table 2). The *Salmonella* test responses to these 38 chemicals range from weakly to strongly positive. The majority (28, 74%) of these mutagenic non-carcinogenic and equivocal chemicals were tested for carcinogenicity prior to 1980, after which the experimental design, pathology, and statistical criteria were changed to reflect what had been learned from the tests conducted tested prior to that time. It is conceivable that if they were re-tested using current protocols, some of the equivocals or non-carcinogens would judged carcinogenic. However, in the absence of such re-testing, the results of these earlier bioassays are accepted as definitive for classification and regulatory purposes.

Of these 38 chemicals, 33 were mutagenic in strains TA98 or TA100 by themselves or in addition to other strains. The five exceptions were 1,2,3-benzotriazole, calcium cyanamide, and 2-chloroethanol which were mutagenic only in TA1535 with S9, 1-chloro-2-propanol which was mutagenic in TA1535 with and without S9 (see [16]), and photodieldrin which was mutagenic only in TA97 with S9.

A mutagenic chemical originally judged an equivocal carcinogen but not listed in Table 2 is *p*-chloroaniline. When administered in feed, *p*-chloroaniline was reported to be an equivocal carcinogen in male rats and male and female mice (TR#189). A subsequent study of *p*-chloroaniline HCl by gavage (TR#351) showed it to be carcinogenic in male rats and mice.

3.1. Structure/activity

Ashby and co-workers [17,18] identified a number of chemical moieties as being 'structurally alerting' when they are part of a larger molecule, that is, these moieties are electrophilic or readily metabolized to electrophiles, and are thereby capable of reacting with the cell's DNA to form pre-mutagenic lesions. With two exceptions, lead dimethyldithiocarbamate (Table 1) and di(*p*-ethylphenyl) dichloroethane (Table 2), all the non-carcinogenic mutagens contain such structural alerts. A few of the alerts appeared to dominate the mutagenic non-carcinogens.

Of the 26 non-carcinogens that were mutagenic, 8 are benzeneamines. There were four $-NO_2$ substituted benzenes, two of which also contained an $-NH_2$ moiety. Four chemicals contain the $-CH_2Cl$ moiety, and three the $-P(=S)-(OR)_2$. Photodieldrin contains an epoxide moiety, and propylene is capable of being metabolized to an epoxide.

Similar distributions were seen among the 12 equivocal carcinogens. There is one benzeneamine and two *N*-substituted benzeneamines. There was one chemical with a phosphothio moiety, one with $-CH_2Cl$, and two that contained free $-NO_2$ groups.

3.2. Phenylenediamines

p-Phenylenediamine and other diamines that are mutagenic in the NTP database have been reported elsewhere as non-mutagenic in *Salmonella* [19,20]. This difference in mutagenic response was attributed to the use of DMSO in lieu of water as the solvent [20], however, other studies [13,21] showed *p*-phenylenediamine to be mutagenic when water was used as the solvent.

Sontag [22] reviewed the NCI rodent carcinogenicity data on the substituted benzenediamines and concluded that five (*p*-phenylenediamine, 2-chloro-*p*-phenylenediamine, 4-nitro-*o*-phenylenediamine, 2,5-diaminotoluene, and 2,6-diaminotoluene) that were judged non-carcinogenic or equivocal were associated with increases in tumors that may have been

Table 3
Carcinogen/non-carcinogen analogues[a]

Carcinogen (TR# or IARC)	SAL	Non-/equivocal-carcinogen (TR#)	SAL
o-Anisidine (#89)	+	*p*-Anisidine (#116) (equivocal)	+
3-Chloromethylpyridine HCl (#95)	+	2-Chloromethylpyridine HCl (#178)	+
4-Chloro-*o*-toluidine HCl (#165)	−	3-Chloro-*p*-toluidine (#145)	+w
5-Chloro-*o*-toluidine (#187)	−		
2-Nitro-*p*-phenylenediamine (#169)	+	4-Nitro-*o*-phenylenediamine (#180)	+
2,6-Dichloro-*p*-phenylenediamine (#219)	+	2-Chloro-*p*-phenylenediamine SO_4 (#113)	+
4-Chloro-*m*-phenylenediamine (#85)	+		
4-Chloro-*o*-phenylenediamine (#63)	+		
2,4-Toluenediamine (#162)	+	2,5-Toluenediamine SO_4 (#126)	+
		2,6-Toluenediamine 2HCl (#200)	+
Chloroform (IARC, 1999)	−	Iodoform (#110)	+
Bromoform (tribromomethane) (#350)	+		
HC Blue 1 (#271)	+	HC Blue 2 (#293)	+
		HC Yellow 4 (#419) (equivocal)	+
Danthron [39] (1,8-dihydroxyanthraquinone)	+w	Emodin (1,3,8-trihydroxy-6-methylanthraquinone) (#493) (equivocal)	+
Ziram (zinc dimethyldithiocarbamate) (#238)	+	Ledate (lead dimethyldithiocarbamate) (#151)	+

[a] SAL: *Salmonella* mutagenicity test result; +: mutagenic; +w: weakly mutagenic; −: not mutagenic. The study abstracts for all Technical Reports (TR) can be found in [10] and/or [11].

treatment-related. These tumors were similar to tumors induced by other phenylenediamines, but the increases were not statistically significant. One possible explanation is that the discrepancies result from mutagenic impurities in the chemicals tested [23,24], another is that these chemicals are weak carcinogens and that the power of the tests was insufficient to conclusively demonstrate their carcinogenicity.

3.3. Structural similarities

A total of 12 mutagenic non-carcinogens and equivocal carcinogens have either carcinogenic analogs or positional isomers that are mutagenic (Table 3).

3.4. 2,4-Toluenediamine (DAT) versus 2,5-diaminotoluene SO_4 and 2,6-diaminotoluene 2HCl

This group of chemicals is probably the best studied of the mutagenic carcinogen/non-carcinogens. The reasons for the distinction between the hepatocarcinogenic 2,4-DAT (TR#162), and its non-carcinogenic structural isomers, 2,5-DAT SO_4 (TR#126) and 2,6-DAT 2HCl (TR#200) are not apparent. 2,4- and 2,6-DAT have been extensively investigated and compared [25–29]. All three chemicals are mutagenic in vitro (Table 1) [12], and both 2,4- and 2,6-DAT induced unscheduled DNA synthesis in primary human hepatocytes in vitro [25], and formed mutagenic metabolites in vivo [26]. 2,4-DAT induced a dose-related increase in cell proliferation in mouse liver, whereas no increase was seen with 2,6-DAT [27]. In mutagenicity studies in vivo using Big Blue® transgenic mice (in which mutation can be measured at the *lac*I transgene), neither 2,4- or 2,6-DAT was mutagenic following 30 days administration in the feed, but 2,4-DAT produced *lac*I mutations in the livers of mice following 90 days exposure [29]. Shorter treatment periods yielded no increases in mutations.

3.5. HC Blue 1 (TR#271), HC Blue 2 (TR#293), and HC Yellow 4 (TR#419)

Different mutagenicity results obtained by different labs testing the carcinogenic dye HC Blue 2 were resolved by the demonstration by Burnett and Corbett [23] and Abu-Shakra et al. [30] that the mutagenicity of HC Blue 2 was due to the presence of low-level, highly mutagenic contaminants. However, when the purified, non-mutagenic HC Blue 2 was tested for carcinogenicity in mice, it retained the same potency it had in the presence of the mutagenic contaminants [23]. HC Blue 1 and HC Yellow 4 (Table 2) are

mutagenic non- and equivocal-carcinogens, respectively, that are structurally similar to HC Blue 2. Experiments to determine whether the mutagenicities of the non-carcinogenic HC Blue 1 and HC Yellow 4 are also the result of low-level mutagenic contaminants have not been reported.

3.6. Propylene and propylene oxide

The non-carcinogen propylene is a weak mutagen in *Salmonella*, whereas its presumptive metabolite, 1,2-propylene oxide, is a mutagen, clastogen [31–33], and rodent carcinogen (TR#267), producing nasal tumors in rats and mice. The absence of carcinogenicity in rats and mice administered propylene may be the result of too low a concentration or rate of formation of the epoxide to be effective, or a rapid elimination of the epoxide by cellular epoxide hydrolases.

3.7. Ziram and ledate

The carcinogen ziram (zinc dimethyldithiocarbamate) (TR#238) and the non-carcinogenic analogue ledate (lead dimethyldithiocarbamate) (TR#151) were both mutagenic [34]. These results suggest that when chelated or delivered by dimethyldithiocarbamate, zinc ions are carcinogenic but lead is not. However, further studies are needed to clarify or explain the different responses.

There are two groupings of chemicals where the non-carcinogen is mutagenic and the carcinogen is non-mutagenic.

3.8. Chloroform, bromoform, and iodoform

The non-mutagenic chloroform [35], and the mutagenic bromoform (tribromomethane) [36] are both carcinogenic ([35], TR#350), whereas the mutagenic iodoform is not (TR#110). The mutagenicity of the three chemicals follows their reactivity. All three chemicals have the potential to form electrophilic carbonium ions. Iodine is the most efficient leaving group, followed by bromine and chlorine. Iodoform is a direct-acting mutagen [34] and bromoform requires metabolic activation for its mutagenicity [37]. Chloroform, which is not mutagenic in vitro, can be metabolized in rats and mice to an electrophilic form [38]; this metabolic pathway may not be effective in vitro. It is presumed that this activation will not occur in the in vitro metabolic activation system used in the mutagenicity test.

3.9. Chloro-toluidines

The non-carcinogenic 3-chloro-*p*-toluidine (TR# 145) is weakly mutagenic, whereas its carcinogenic congeners, 4-chloro-*o*-toluidine (TR#165), and 5-chloro-*o*-toluidine (TR#187) are not mutagenic. There have been no reported studies to investigate or explain this pattern of responses.

3.10. Other mutagenic carcinogen/non-carcinogen pairs or triplets (Table 3)

3-Chloromethylpyridine HCl (TR#95)/2-chloromethylpyridine HCl (TR#178), 2-nitro-*p*-phenylenediamine (TR#169)/4-nitro-*o*-phenylenediamine (TR# 180), 4-chloro-*m*-phenylenediamine (TR#85) and 4-chloro-*o*-phenylenediamine (TR#63) and 2,6-dichloro-*p*-phenylenediamine (TR#219)/2-chloro-*p*-phenylenediamine SO_4 (TR#113), *o*-anisidine HCl (TR#89)/*p*-anisidine HCl (TR#116), emodin (TR# 493)/danthron [39], have not been extensively investigated.

3.11. Other genetic effects

The high sensitivity engineered into the *Salmonella* tester strains to enhance their ability to detect DNA damage leading to mutagenicity [1] does not appear to contribute to the frequency of mutagenic non-carcinogens. Chemicals mutagenic in *Salmonella* also tended to yield positive responses in mammalian cell tests; they produced chromosome aberrations (ABS) and sister-chromatid exchanges (SCE) in CHO cells, and mutations in mouse lymphoma cells (MLA).

Data are available for 35 non-carcinogenic *Salmonella* mutagens tested in other genetic toxicity tests (Tables 1, 2 and 4). Of these, all but four chemicals were positive in at least one other in vitro test. As a rule, if the *Salmonella* test was positive, the chemical was positive in the other in vitro tests, regardless of whether it was a carcinogen or non-carcinogen. Of the 19 non-carcinogenic chemicals tested in all four in vitro tests (SAL, ABS, SCE, MLA), 12 (63%)

Table 4
Agreement between positive responses in *Salmonella* and in other in vitro short-term tests[a]

	ABS+/SAL+		SCE+/SAL+		MLA+/SAL+	
Non-carcinogens	0.58	14/24[b]	0.84	21/25	0.72	13/18
Equivocal carcinogens	0.67	6/9	1.00	8/8	1.00	2/2
Carcinogens	0.71	44/62	0.84	52/62	0.94	48/51

[a] ABS: chromosome aberrations in CHO cells in vitro; SCE: sister-chromatid exchanges in CHO cells in vitro; MLA: mouse lymphoma L5178Y mutation assay; SAL: mutation in the *Salmonella* test.

[b] Number of chemicals positive in the designated test/number positive in *Salmonella*, adapted from [12].

were positive in all four. The high correlation between *Salmonella* mutagens and positive results in the other tests has previously been noted with respect to carcinogens [36,40,41].

The majority of mutagenic non-carcinogens (88%) and equivocal carcinogens (67%) were within the initial 200 chemicals tested. These chemicals were put on test in 1972–1974, and the high proportion of *Salmonella* false-positives could reflect the low sensitivity of the carcinogenicity test protocols and data evaluation procedures in use at the time, or could also be a function of the chemicals selected for testing [42]. As noted above, the majority of these chemicals contain structural alerts, indicating that they contain electrophilic centers that can react with (alkylate) DNA. The question that cannot be answered without additional research is why these DNA-reactive agents are not carcinogenic in lifetime rodent studies.

4. Discussion

The *Salmonella* mutagenicity test is most often performed as a component of the safety and hazard evaluation process by industrial and regulatory organizations. A chemical that is mutagenic in *Salmonella* is presumed to be a rodent carcinogen (and potential human carcinogen). Many chemicals found early in their commercial development to be mutagenic are not developed further, because the cost to industry and society of challenging the presumption of carcinogenicity from a positive *Salmonella* mutagenicity test is prohibitive in cost and time. The only way to overcome this presumption is to perform a 2 year carcinogenicity test with the suspect chemical in mice and rats that results in a judgement of non-carcinogenicity, or to demonstrate that the *Salmonella* test results were not relevant.

The term "false-positive", when applied to a *Salmonella* mutagenicity test result is a misnomer. The *Salmonella* test measures the ability of a chemical to induce mutations, not cancer. Therefore, a positive result in a *Salmonella* test is a true measure of the ability of the test chemical to induce gene mutations. The discordance of such a response with the rodent cancer test result raises the questions of whether that in vitro mutagen would also be mutagenic in vivo, and whether the rodent cancer response was "true" or "false". The *Salmonella* mutagenicity test, when performed by experienced laboratories using standardized protocols, has a reproducibility of 84–87% [43]. Because of the absence of comparable data from the rodent carcinogenicity test, the reproducibility of that test, and consequently, its proportions of false (i.e. unrepeatable) positives and negatives, are not known.

Whether the false-positive rate in the *Salmonella* test (10–30%) is too high or too low depends on the decisions that the *Salmonella* test result will support, and the standard against which the rate is being compared. If the test has a low false-positive rate the chemical can be discarded without much concern that a potentially useful, non-hazardous chemical is being lost. However, if the false-positive rate is high, many potentially useful chemicals may be unjustly discarded as potential carcinogens, leading to a potential cost to society of lost benefits.

The false-positive result cannot be reduced simply be redefining the requirements for a positive response. Prival and Dunkel [9] performed a detailed analysis of the mutagenicity and carcinogenicity of 25 NCI/NTP chemicals identified as "false-positives", which are among those addressed here. When they

made the criteria for a positive mutagenic response more stringent, they succeeded in reducing the number of "false" positives but at the cost of an equivalent loss of "true" positives.

There are many explanations that could account for, or explain, the existence of mutagenic non-carcinogens. These include: metabolic differences between the in vivo carcinogenicity test and in vitro *Salmonella* mutation test conditions; the differences between the effective dose in vitro, and the in vivo distribution of the agent and its effective dose; the possibility that low levels of mutagenic contaminants (which may not reach a sufficient concentration to produce tumors) are responsible for the *Salmonella* response; differences in DNA repair among species, and; the relative sensitivities of the bacterial and rodent systems to genetic damage. The mechanistic explanation for the absence of carcinogenicity would be expected to be different for different chemical classes, and perhaps for the individual members of the class. It is not the intent of this report to determine whether the mutagenic response in vitro is unique to the in vitro protocol, or to explore the various mechanisms that may be operating to intercept the carcinogenic process in rodents.

The in vitro mutagenicity tests have often been characterized as too sensitive to be used as accurate predictors of carcinogens and non-carcinogens. The high sensitivity engineered into the *Salmonella* tester strains to enhance their ability to detect DNA damage leading to mutagenicity [1] does not appear to contribute to the frequency of mutagenic non-carcinogens. The same chemicals tend to yield positive responses in mammalian cell tests. The proportions of *Salmonella* positive non-carcinogens that are also positive in The ABS and SCE test are equal to the proportions obtained when carcinogens are tested (Table 4).

Regardless of the rationale used to explain the non-carcinogenicity of chemicals that clearly are electrophilic mutagens, the mutagenicity test is identifying chemicals that are potentially hazardous. Whether the lack of in vivo activity reflects basic differences between the mechanisms of mutagenicity in these *Salmonella* strains and the mechanisms of mutagenicity in the potential target organs of mice and rats, or is a result of a dilution or in vivo metabolic "deactivation" of the chemicals, may not be relevant. The important point is that the chemicals have a structure and in vitro chemical activity that is associated with carcinogenesis in animals.

5. Summary

The presence of mutagenic non-carcinogens does not detract from the tests or from the theory. The "false" positive results in *Salmonella* are not a function of the bacterial strains or test procedure because, when tested in mammalian cells in vitro, the majority these same chemicals were mutagenic and/or clastogenic. Mutagenicity, and subsequent carcinogenicity, in vivo requires the active mutagenic chemical, usually an electrophile, to reach an appropriate target cell in sufficient concentration to induce a DNA lesion that is converted to a mutation. The mutagenicity tests measure the intrinsic ability of the test chemical or its metabolites to react with DNA to produce a pre-mutagenic lesion. Unlike mutation in bacteria, carcinogenicity in mammals is a multi-step phenomenon, of which, gene mutation may be only a single step. After a mutation is induced in a cell, additional replication cycles and changes are needed before the mutated cell becomes a tumor cell, which must then survive within the body and replicate to form a tumor. The carcinogenicity test identifies only those chemicals that can induce tumor cells that survive and replicate to form visible tumors, whereas the mutagenicity test identifies chemicals that react with cellular DNA to produce mutations.

References

[1] D.M. Maron, B.N. Ames, Revised methods for the *Salmonella* mutagenicity test, Mutat. Res. 113 (1983) 173–215.

[2] J. McCann, E. Yamasaki, B.N. Ames, Detection of carcinogens in the *Salmonella*/microsome test: assay of 300 chemicals, Proc. Natl. Acad. Sci. U.S.A. 72 (1975) 5135–5139.

[3] T. Sugimura, S. Sato, M. Nagao, T. Yahagi, T. Matsushima, Y. Seino, M. Takeuchi, T. Kawachi, Overlapping of carcinogens and mutagens, in: P.N. Magee, S. Takayama, T. Sugimura, T. Matsushima (Eds.), Fundamentals of Cancer Prevention, University Park Press, Baltimore, 1976, pp. 191–215.

[4] I.F.H. Purchase, E. Longstaff, J. Ashby, J.A. Styles, D. Anderson, P.A. Lefevre, F.R. Westwood, An evaluation of 6 short-term tests for detecting organic chemical carcinogens, Br. J. Cancer 37 (1978) 873–959.

[5] L.D. Kier, D.J. Brusick, A.E. Auletta, E.S. Von Halle, V.F. Simmon, M.M. Brown, V.C. Dunkel, J. McCann, K.

Mortelmans, M.J. Prival, T.K. Rao, V.A. Ray, The *Salmonella typhimurium*/mammalian microsome mutagenicity assay. A report of the US Environmental Protection Agency Gene-Tox program, Mutat. Res. 168 (1986) 67–238.

[6] E. Zeiger, Identification of rodent carcinogens and non-carcinogens using genetic toxicity tests: premises, promises, and performance, Regul. Toxicol. Pharmacol. 28 (1998) 85–95.

[7] M.D. Shelby, S. Stasiewicz, Chemicals showing no evidence of carcinogenicity in long-term, two-species rodent studies: the need for short-term test data, Environ. Mutagen. 6 (1984) 871–878.

[8] J. Ashby, I.F.H. Purchase, Significance of the genotoxic activities observed in vitro for 35 of 70 NTP non-carcinogens, Environ. Mutagen. 7 (1985) 747–758.

[9] M.J. Prival, V.C. Dunkel, Re-evaluation of the mutagenicity and carcinogenicity of chemicals previously identified as false-positives in the *Salmonella typhimurium* mutagenicity assay, Environ. Mol. Mutagen. 13 (1989) 1–24.

[10] J.K. Selkirk, S.M. Soward, Compendium of abstracts from long-term cancer studies reported by the National Toxicology Program from 1976 to 1992, Environ. Health Perspect. 101 (Suppl. 1) (1993) 3–281.

[11] EHIS (Environmental Health Information Service), NTP Reports, Toxicology and Carcinogenesis Studies, 2000, http://ehis.niehs.nih.gov/ntp/docs/ntp.htmltech.

[12] E. Zeiger, Genotoxicity database, in: L.S. Gold, E. Zeiger (Eds.), Handbook of Carcinogenic Potency and Genotoxicity Databases, CRC Press, Boca Raton, 1997, pp. 687–729.

[13] E. Zeiger, B. Anderson, S. Haworth, T. Lawlor, K. Mortelmans, *Salmonella* mutagenicity tests. IV. Results from the testing of 300 chemicals, Environ. Mol. Mutagen. 11 (Suppl. 12) (1988) 1–157.

[14] R.S. Chhabra, J.E. Huff, B.S. Schwetz, J. Selkirk, An overview of pre-chronic and chronic toxicity/carcinogenicity experimental designs and criteria used by the National Toxicology Program, Environ. Health Perspect. 86 (1990) 313–321.

[15] J.E. Huff, J.K. Haseman, E.E. McConnell, J.A. Moore, The National Toxicology Program toxicology data evaluation techniques, and long-term carcinogenesis studies, in: W.E. Lloyd (Ed.), Safety Evaluation of Drugs and Chemicals, Hemisphere, Washington, DC, 1986, pp. 411–446.

[16] M.J. Prival, E. Zeiger, Chemicals mutagenic in *Salmonella typhimurium* strain TA1535 but not in TA100, Mutat. Res. 412 (1998) 251–260.

[17] J. Ashby, Fundamental structural alerts to potential carcinogenicity or non-carcinogenicity, Environ. Mutagen. 7 (1985) 919–921.

[18] J. Ashby, R.W. Tennant, Definitive relationships among chemical structure, carcinogenicity, and mutagenicity for 301 chemicals tested by the US NTP, Mutat. Res. 257 (1991) 229–306.

[19] M.M. Shahin, P. Andrillon, N. Goetz, P. Bore, A. Bugaut, G. Kalopissis, Studies on the mutagenicity of *p*-phenylenediamine in *Salmonella typhimurium*. Presence of PCB's in rat-liver microsomal fraction induced by Aroclor, Mutat. Res. 68 (1979) 327–336.

[20] C. Burnett, C. Fuchs, J. Corbett, J. Menkart, The effect of dimethylsulfoxide on the mutagenicity of the hair dye *p*-phenylenediamine, Mutat. Res. 133 (1982) 1–4.

[21] V.C. Dunkel, E. Zeiger, D. Brusick, E. McCoy, D. McGregor, K. Mortelmans, H.S. Rosenkranz, V.F. Simmon, Reproducibility of microbial mutagenicity assays. II. Testing of carcinogens and non-carcinogens in *Salmonella typhimurium and Escherichia coli*, Environ. Mutagen. 7 (Suppl. 5) (1985) 1–248.

[22] J.M. Sontag, Carcinogenicity of substituted-benzenediamines (phenylenediamines) in rats and mice, J. Nat. Cancer Inst. 66 (1981) 591–602.

[23] C.M. Burnett, J.F. Corbett, Failure of short-term in vitro mutagenicity tests to predict the animal carcinogenicity of hair dyes, Fd. Chem. Toxicol. 25 (1987) 703–707.

[24] M.M. Shahin, A. Bugaut, G. Kalopissis, Structure-activity relationship within a series of *m*-diaminobenzene derivatives, Mutat. Res. 78 (1980) 25–31.

[25] B.E. Butterworth, T. Smith-Oliver, L. Earle, D.J. Loury, R.D. White, D.J. Doolittle, P.K. Working, R.C. Cattley, R. Jirtle, G. Michalopoulos, S. Strom, Use of primary cultures of human hepatocytes in toxicology studies, Cancer Res. 49 (1989) 1075–1084.

[26] M.L. Cunningham, L.T. Burka, H.B. Matthews, Metabolism, disposition, and mutagenicity of 2,6-diaminotoluene, a mutagenic non-carcinogen, Drug Metab. Dispos. 17 (1989) 612–617.

[27] M.L. Cunningham, J. Foley, R.R. Maronpot, H.B. Matthews, Correlation of hepatocellular proliferation with hepatocarcinogenicity induced by the mutagenic non-carcinogen:carcinogen pair-2,6- and 2,4-diaminotoluene, Toxicol. Appl. Pharmacol. 107 (1991) 562–567.

[28] M.L. Cunningham, J.J. Hayward, B.S. Shane, K.R. Tindall, Distinction of mutagenic carcinogens from a mutagenic non-carcinogen in the Big Blue transgenic mouse, Environ. Health Perspect. 104 (Suppl. 3) (1996) 683–686.

[29] J.J. Hayward, B.S. Shane, K.R. Tindall, M.L. Cunningham, Differential in vivo mutagenicity of the carcinogen/non-carcinogen pair 2,4- and 2,6-diaminotoluene, Carcinogenesis 16 (1995) 2429–2433.

[30] A. Abu Shakra, L. Johnson, K. Earley, C.W. Jameson, F.W. Kari, R. Gupta, R. Langenbach, Isolation of the mutagenic and DNA adduct-inducing components from a commercial preparation of HC Blue 1 using *Salmonella* (TA98) bioassay-directed HPLC fractionation, Mutat. Res. 260 (1991) 377–385.

[31] D.A. Canter, E. Zeiger, S. Haworth, T. Lawlor, K. Mortelmans, W. Speck, Comparative mutagenicity of aliphatic epoxides in *Salmonella*, Mutat. Res. 172 (1986) 105–138.

[32] D.K. Gulati, K. Witt, B. Anderson, E. Zeiger, M.D. Shelby, Chromosome aberration and sister-chromatid exchange tests in Chinese hamster ovary cells in vitro. III. Results with 27 chemicals, Environ. Mol. Mutagen. 13 (1989) 133–193.

[33] D. McGregor, A.G. Brown, P. Cattanach, I. Edwards, D. McBride, C. Riach, W. Shepherd, W.J. Caspary, Responses of the L5178Y mouse lymphoma forward mutation assay. V. Gases and vapors, Environ. Mol. Mutagen. 17 (1991) 122–129.

[34] S. Haworth, T. Lawlor, K. Mortelmans, W. Speck, E. Zeiger, *Salmonella* mutagenicity test results for 250 chemicals, Environ. Mutagen. 5 (Suppl. 1) (1983) 3–142.

[35] IARC, IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, Some Chemicals that Cause Tumours of the Kidney or Urinary Bladder in Rodents and Some Other Substances, Vol. 73, International Agency for Research on Cancer, Lyon, 1999, pp. 132–182.

[36] E. Zeiger, J.K. Haseman, M.D. Shelby, B.H. Margolin, R.W. Tennant, Evaluation of four in vitro genetic toxicity tests for predicting rodent carcinogenicity: confirmation of earlier results with 41 additional chemicals, Environ. Mol. Mutagen. 16 (Suppl. 18) (1990) 1–14.

[37] E. Zeiger, Mutagenicity of 42 chemicals in *Salmonella*, Environ. Mol. Mutagen. 16 (Suppl. 18) (1990) 32–54.

[38] L. Vittozzi, S. Gemma, M. Sbraccia, E. Testai, Comparative characterization of $CHCl_3$ metabolism and toxicokinetics in rodent strains differently susceptible to chloroform-induced carcinogenicity, Environ. Toxicol. Pharmacol. 8 (2000) 103–110.

[39] IARC, IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, Some Pharmaceutical Drugs, Vol. 50, International Agency for Research on Cancer, Lyon, 1990, pp. 265–273.

[40] R.W. Tennant, B.H. Margolin, M.D. Shelby, E. Zeiger, J.K. Haseman, J. Spalding, W. Caspary, M. Resnick, S. Stasiewicz, B. Anderson, R. Minor, Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays, Science 236 (1987) 933–941.

[41] R. Benigni, Analysis of the National Toxicology Program data on in vitro genetic toxicity tests using multivariate statistical methods, Mutagenesis 4 (1989) 412–419.

[42] V.A. Fung, J. Huff, E.K. Weisburger, D.G. Hoel, Predictive strategies for selecting 379 NCI chemicals evaluated for carcinogenic potential: scientific and public health impact, Fundam. Appl. Toxicol. 20 (1993) 413–436.

[43] W.W. Piegorsch, E. Zeiger, Measuring intra-assay agreement for the Ames *Salmonella* assay, in: L. Hothorn (Ed.), Statistical Methods in Toxicology, Lecture Notes in Medical Informatics, Vol. 43, Springer, Heidelberg, 1991, pp. 35–41.