From: "James R. Coughlin"
To: "Cynthia Oshita " <COSHITA@oehha.ca.gov>
CC: <jmurray2@sbcglobal.net>
Date: 5/5/2009 4:43 PM
Subject: Comments by IMOA on Molybdenum Trioxide for the CIC Prioritization Meeting May 29
Attachments: #1011 CTL Ames 2003-IMOA.pdf; #1012 CTL IVMN 2004-IMOA inc FC.pdf; Scott et 1991.pdf; #0121-Huvinen_2002.pdf; #1567_Huvinen_1996.pdf; Prop 65_IMOA Comments_May 5 2009.pdf

Dear Cindy,

Attached are comments (in pdf format) being submitted by the International Molybdenum Association (IMOA) on "Molybdenum Trioxide" for the CIC Prioritization Meeting scheduled for May 29. I am also attaching some key references (in pdf format) cited in our comments that were not included in OEHHA's Summary Document on Molybdenum Trioxide, so that they can be made available to CIC members upon request.

If you have any questions or need additional information, please don't hesitate to contact me or Jay Murray, since the IMOA folks are located in the UK.

Thank you for your attention to our submission.

Best regards,

Jim

James R. Coughlin, Ph.D.

President, Coughlin & Associates:

Consultants in Food/Chemical/Environmental Toxicology and Safety

27881 La Paz Road, Suite G, PMB 213

Laguna Niguel, CA 92677

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INTERNATIONAL MOLYBDENUM ASSOCIATION

4 Heathfield Terrace
Chiswick
London
W4 4JE

Tel: 0207 871 1580
Fax: 0208 994 6067
Email:info@imoa.info
Website: www.imoa.info

May 5, 2009

Members of the Carcinogen Identification Committee

Cynthia Oshita
Office of Environmental Health Hazard Assessment
Proposition 65 Implementation
P.O. Box 4010
1001 I Street, 19th floor
Sacramento, California 95812-4010

Re: Prioritization of Molybdenum Trioxide

Dear Chairperson Mack, Members of the Carcinogen Identification Committee, and Ms. Oshita:

On behalf the International Molybdenum Association (**IMOA**), we are writing to recommend that molybdenum trioxide be given a **Low Priority** for further carcinogenicity review. IMOA was founded in 1989 and is registered under Belgian law as a non-profit trade association (ASBL) with scientific purposes. IMOA's current membership of 68 companies represents 85% of Western World production and all conversion facilities; the largest mines in China are also members, together with Chinese converters. Health, Safety & Environment, and Market Development are the core IMOA activities. Along with this submission, we are also providing electronic copies of key references we cite herein that were not included in the OEHHA Summary Document, so that they can be made available to CIC members upon request.

EXECUTIVE SUMMARY

Molybdenum trioxide should be given a Low Priority for the following reasons.

1. **No Authoritative Body has Classified Molybdenum Trioxide as Causing Cancer:** Molybdenum trioxide (MoO_3)(CAS No. 1313-27-5) has not been formally identified as causing cancer by any of Proposition 65's five Authoritative Bodies, including the National Toxicology Program (NTP). Molybdenum trioxide does not meet the "Sufficient Evidence" of carcinogenicity criteria required by an Authoritative Body listing. The NTP has conducted and reported a chronic inhalation carcinogenicity

bioassay of molybdenum trioxide only showing "Some Evidence" of carcinogenicity in the mouse lung. Consequently, the NTP study results do not support an Authoritative Body listing.

2. **Exposure and Uses:** OEHHA noted that exposure to molybdenum trioxide is "limited/occupational." Actual molybdenum trioxide exposure to the California public/consumers is very limited and insignificant. The majority of molybdenum trioxide sold into California is used in the manufacture of petroleum catalysts by one or two companies. This catalyst is then shipped out of state to be activated, during which process the molybdenum trioxide is converted to molybdenum sulfide. The uses stated by OEHHA actually cover all molybdenum chemicals, not just molybdenum trioxide, and will be separately addressed below.

3. **Animal Carcinogenicity Data (NTP, 1997, 1998):** The key findings of the NTP 2-year bioassay of molybdenum trioxide in rats and mice do not provide "Sufficient Evidence" of a carcinogenic effect:

    a. Male rats provided only "Equivocal Evidence" of carcinogenicity and female rats provided "No Evidence" of carcinogenicity.

    b. Only "Some Evidence" of carcinogenicity in the lung was reported by NTP for male and female mice, but these findings did not reach NTP's highest category of "Clear Evidence."

    c. "Biological plausibility" and statistical significance arguments based on studies and criteria published by NTP's retired Chief of the Biostatistics Branch, Dr. Joseph Haseman, are not satisfied for the carcinogenicity of molybdenum trioxide in the mouse [see Appendix II]:

        i. Male Mouse Lung Tumors: no statistically significant increase was reached in adenomas at any dose; there was no dose-response in the incidence of carcinomas; the high-dose carcinomas did not reach the required $P < 0.01$ needed for a common tumor; the combined adenomas or carcinomas only reached statistical significance at the required $P < 0.01$ for the low dose.

        ii. Female Mouse Lung Tumors: adenomas were not statistically significantly increased at the required $P < 0.01$ at any dose; no statistically significant increase was observed in carcinomas at any dose; the combined adenomas or carcinomas only reached statistical significance at the required $P < 0.01$ for the high dose.

    d. The finely micronized molybdenum trioxide product tested by NTP is not encountered in industrial or consumer exposures. Its micronization by NTP, producing a test substance from 1.3 - 1.5 μm particle size, resulted in over 600 times greater exposure of these particles to the mouse lower lung than if the actual, undensified molybdenum trioxide "Form A" itself had been used in the bioassay.

4. **Animal Carcinogenicity Data (Stoner et al., 1976):** This short-term, high-dose intraperitoneal injection study in mice provides no useful carcinogenicity information on molybdenum trioxide.

5. **Epidemiological Data:** The one epidemiological study claiming to be a positive occupational study of lung cancer (Droste et al., 1999) is based upon an examination of many mixed exposures to various substances, not just to molybdenum trioxide exposure, and is considered to be a poorly conducted study.

6. **Genotoxicity Data:** Molybdenum trioxide is non-genotoxic in the NTP assays and also in three assays conducted for IMOA by the Central Toxicology Laboratory, UK (CTL). Some studies cited by OEHHA that purport to demonstrate positive genotoxicity effects are either studies of molybdenum compounds other than molybdenum trioxide or are deficient because of methodological flaws, particularly when the addition of molybdenum trioxide is known to reduce the pH of the assay systems' culture media and give false-positive effects due to the lowered pH.

7. **Human and Plant Essentiality of Molybdenum:** Molybdenum is an essential trace element with a firmly established RDA for humans (FNB, 2001) and is also essential for other mammals and plants. Molybdenum exposure occurs naturally as an essential nutrient in foods, as an added nutrient in vitamin/mineral supplements (as sodium molybdate, not molybdenum trioxide) and from other molybdate forms, such as sodium molybdate, as a fertilizer in deficient soils. Therefore, it would not serve any California public health interests to list a human and plant essential trace nutrient as a carcinogen under Proposition 65.

1. NO AUTHORITATIVE BODY HAS CLASSIFIED MOLYBDENUM TRIOXIDE AS CAUSING CANCER

Molybdenum trioxide (MoO_3) has not been formally identified as causing cancer by any of Proposition 65's five Authoritative Bodies, including the National Toxicology Program (NTP). OEHHA pointed out in their background prioritization materials released on March 5, 2009 that they applied two data screens (animal and human) to roughly half the chemicals in a tracking database of chemicals to which Californians are potentially exposed. Molybdenum trioxide was one of the chemicals that "passed" OEHHA's animal data screen and was therefore included in the notice requesting public comments.

OEHHA pointed out, however, that "Candidate chemicals that are candidates for listing via an administrative listing mechanism were not screened." We agree with OEHHA that molybdenum trioxide does not qualify as a candidate for Authoritative Body listing, since it does not meet the listing criteria of "Sufficient Evidence" of carcinogenicity required by an Authoritative Body listing (*27 CCR Section 25306*).

2. EXPOSURE AND USES

Uses of Molybdenum Compounds in California.

As noted by OEHHA in its Summary Table, molybdenum trioxide has only "Limited/occupational" exposure. Molybdenum trioxide does not have widespread industrial use within the State of California, and based upon the limited use of molybdenum trioxide within the State, occupational exposures are expected to be minimal as well. This conclusion is based upon many years of commercial experience by IMOA's member companies within the State of California. These member companies account for most, if not all, of the molybdenum trioxide transported into the State.

Of the uses for molybdenum trioxide that are cited by OEHHA in the "Molybdenum Trioxide" summary document, the following review for each cited use is provided:

- *"Its major use is as an additive to steel and other corrosion-resistant alloys."* In fact, though molybdenum trioxide is a component of specialty steels and corrosion resistant alloys, we are not aware of any such production of these alloys in California. For specialty steels that are produced out of state and shipped into California, once the molybdenum trioxide is incorporated into these alloy and specialty steel products, the chemical form of the molybdenum is converted to a metallic alloy and no longer is molybdenum trioxide.

- *"It is also used in the production of molybdenum products."* In fact, the same analysis presented above for specialty steels and alloys applies to the use of molybdenum trioxide for the production of molybdenum metal products. In this process, molybdenum oxide is reduced by hydrogen in small furnaces to the metal form of molybdenum. The metallic powder can then be converted to other product shapes. We are not aware of any such industrial activities within California. In addition, with molybdenum products shipped into California, the molybdenum trioxide no longer exists, since it was converted to metallic molybdenum.

- *"…as an industrial catalyst"* In fact, the single largest use of pure molybdenum trioxide is as a component in the manufacture of hydrodesulfurization catalysts, and we are only aware of one major company within the State that produces this catalyst. In this manufacturing process, the molybdenum trioxide is incorporated along with other metals into a catalyst matrix such as alumina. Once impregnated into the catalyst, the catalyst is shipped out of state where it is activated at high temperature in a reducing atmosphere, under which conditions the molybdenum trioxide is converted to a sulfide. The final, activated catalyst is then placed into reactor vessels at petroleum refineries to convert sulfur to a gaseous form that can be collected and recovered. Exposures during manufacture of the catalyst are well below state industrial hygiene standards, and exposure once in use in refineries is negligible, since the molybdenum trioxide is no longer present.

- *"a pigment"* Ammonium octamolybdate is the form of molybdenum that is used in the pigment industry. As a result, there will not be any use of or exposure to molybdenum trioxide in this industry.

- *"a crop nutrient"* The chemical form of molybdenum that is used as a crop nutrient supplement (fertilizer) is sodium molybdate and not molybdenum trioxide. This is due to the more neutral pH properties of sodium molybdate. As a

result, there will not be any use of or exposure to molybdenum trioxide as a crop nutrient.

- *"a component of glass, ceramics, and enamels"* It is possible that some form of molybdenum could be used in these applications; however, we are not aware of any such use of molybdenum trioxide for these applications. Furthermore, after firing of the glass, ceramic or enamel, the form of the molybdenum will be converted to a non oxide derivative of molybdenum. Under these circumstances and use, there will not be any exposure to molybdenum trioxide.

- *"a flame retardant"* Ammonium octamolybdate is the chemical form of molybdenum that is used in flame retardant and smoke suppressant applications. Consequently, there will not be any use of or exposure to molybdenum trioxide under this use.

- *"and as a chemical reagent"* IMOA is not aware of any significant uses of molybdenum trioxide as a chemical reagent in California other than as described in the above applications. Therefore, exposures in this area are insignificant.

3. ANIMAL CARCINOGENICITY DATA (NTP, 1997, 1998)

There are three different forms of molybdenum trioxide, and the form tested in the NTP bioassay is not the form to which people are exposed. The NTP published a carcinogenesis bioassay study (NTP Technical Report No. 462, 1997) on molybdenum trioxide in 1997, and the study was subsequently published in the literature (Chan et al., 1998). It was a standard NTP toxicology and carcinogenesis study using F344/N rats and B6C3F1 mice (inhalation studies) of undensified sublimed pure molybdenum trioxide, which has the smallest particle size of the three forms of molybdenum trioxide commercially produced (**see Appendix I, "Form A" of molybdenum trioxide in the table**).

The commercially-produced "Form A" molybdenum trioxide that was provided to NTP for testing had a volume mean diameter of 39 µm, much smaller than the commercially-produced "Form B" (262 µm) or "Form C" (185 µm). However, it is critically important to point out that for the purpose of the two-year inhalation bioassay, the NTP micronized (or air milled) this undensified sublimed pure oxide ("Form A") to even further reduce the average particle size, ranging from 1.3 - 1.5 µm for the mouse bioassay and 1.5 - 1.7 µm for the rat bioassay. The test product thus obtained and tested was a form of the chemical which is neither commercially available nor would exist during the manufacturing process or during normal handling and use. This "Form A" product is not sold commercially in any significant quantities into California, and California workers and the public cannot possibly be exposed to the further micronized product tested by NTP. In fact, the majority of molybdenum trioxide sold into California is used in the manufacture of petroleum catalysts by one or two companies, during which process the molybdenum trioxide is converted to molybdenum sulfide.

The NTP Study findings throughout the respiratory tract in both rats and mice at termination of the lifetime studies were consistent with exposure to a direct-acting irritant aerosol. This, in turn, is consistent with the low pH (2.4) of the test material in solution, where the resulting acidity during solubilization at the sites of deposition, in association with prolonged

inhalation exposure, would lead to the development of the non-neoplastic responses seen in both species in the nose, larynx and lung. It is well known that larger-sized particles (> 5 μm) are deposited primarily in the upper respiratory tract, whereas much finer particles are deposited in the small airways and alveoli. Thus, the very fine, micronized particles (< 2 μm) of molybdenum trioxide to which the animals were exposed resulted in over 600 times greater exposure to these particles in the lower lung than if the "Form A" molybdenum trioxide (39 μm) had been used in the bioassay.

This evidence indicates that the results of the NTP lifetime study of mice provide no determination of "Clear Evidence" of carcinogenicity. NTP itself actually concluded that there was only "Some Evidence" in the male mouse and female mouse based on the increased incidence of lung tumors, and there was no reported increase of any tumors in the upper respiratory tract. When the patterns and incidences of lung tumors observed in the treated mice are considered in the context of the already high spontaneous background incidence of lung tumors in the B6C3F1mouse, this further supports that classification of molybdenum trioxide as an animal carcinogen is not scientifically supportable, nor is the listing of molybdenum trioxide as a carcinogen under Proposition 65.

In addition, the NTP reported that the findings in F344/N female rats in the 2-year bioassay provided "No Evidence" of carcinogenicity and the findings in male rats provided only "Equivocal Evidence" of carcinogenicity. NTP termed the male rat's finding of "Equivocal Evidence" as "Uncertain Findings" in the NTP Abstract's Summary Table (page 8), since statistically the increase in lung tumors produced in male rats was only a marginally significant positive trend.

In **Appendix II**, there is a brief background review on the "Statistical and Biological Significance of NTP Cancer Bioassay Findings." This review is based on published articles by Dr. Joseph K. Haseman, who was the NIEHS/NTP Chief of the Biostatistics Branch and Director of Statistical Consulting during his 33-year career there (he retired in 2004). Dr. Haseman was primarily responsible for the experimental design and data analysis of the NTP rodent carcinogenicity program. Many of his papers concerned the statistical design and interpretation of NTP cancer bioassay results, which provide an understanding of how NTP uses statistically significant findings in interpreting its cancer bioassays.

Haseman has written often about a statistical decision rule that closely approximates the scientific judgment process used to evaluate the NTP studies:

> "Declare a compound carcinogenic if any common tumor showed a significant ($P < 0.01$) high-dose effect or if a $P < 0.05$ high-dose effect occurred for an uncommon tumor."

Said another way, Haseman's decision rule was described as follows:

> "…regard as carcinogenic any chemical that produces a high-dose increase in a common tumor that is statistically significant at the 0.01 level or a high-dose increase in an uncommon tumor that is statistically significant at the 0.05 level."

In essence, the key points made by Haseman were to be aware that it is <u>not</u> appropriate to blindly regard every $P < 0.05$ statistically positive finding as a biological positive, and that when a common tumor is being evaluated, such as the lung tumors seen for molybdenum

trioxide in the B6C3F1 mouse, the finding must reach the $P < 0.01$ level of significance if it is to have any potential biological relevance.

With regard to the NTP Study results for molybdenum trioxide in the male and female mouse lung (see Table 4 in Chan et al., 1998), it is important to point out that some of the specific lung tumor findings not only failed to give an increasing dose-response, but some also did not reach the $P < 0.01$ level of significance required for a common tumor like the mouse lung tumor:

1. Male Mouse Lung Tumors:
    a. no statistically significant increase was reached in adenomas at any dose;
    b. there was no dose-response in the incidence of carcinomas;
    c. the high-dose carcinomas did not reach the required $P < 0.01$ needed for a common tumor;
    d. the combined adenomas or carcinomas only reached statistical significance at the required $P < 0.01$ for the low dose, but did not at the mid or high dose.

2. Female Mouse Lung Tumors:
    a. adenomas were not statistically significantly increased at the required $P < 0.01$ at any dose;
    b. no statistically significant increase was observed in carcinomas at any dose;
    c. the combined adenomas or carcinomas only reached statistical significance at the required $P < 0.01$for the high dose.

Given Dr. Haseman's decision rules on both dose-response and statistical significance described above, it is clear that the lung tumor findings for molybdenum trioxide in the NTP male and female mice present very weak evidence to conclude that molybdenum trioxide is a mouse lung carcinogen.

4. ANIMAL CARCINOGENICITY DATA (Stoner et al., 1976)

Stoner et al. (1976) published a short-term, high-dose intraperitoneal injection study of molybdenum trioxide. Groups of 20 strain A mice (10 males and 10 females) received thrice-weekly intraperitoneal (i.p.) injections of 0.85% saline control or 50, 125 or 200 mg molybdenum trioxide/kg bw (total of 19 injections). A single i.p. injection of 20 mg urethane/mouse served as positive control. Mice were sacrificed 30 weeks after the first injection, their lungs were removed and fixed. After 1 to 2 days, the milky-white nodules on the lungs were counted, and a few nodules were examined histopathologically to confirm the typical morphological appearance of adenoma, a benign tumor. Only the highest total dose of 4,750 mg of molybdenum trioxide per kg mouse bw resulted in a statistically significant ($p<0.05$) increase in the average number of lung adenomas per mouse. No tumors other than lung adenoma were observed. Results of the histological examination of other organs (liver, intestines, thymus, kidney, spleen, salivary, and endocrine glands) were not reported.

The results of this short-term, high-dose, i.p. injection bioassay of molybdenum trioxide do not provide any meaningful or supporting information on the carcinogenicity of the chemical. In addition, this route of exposure is totally irrelevant to assessing the potential carcinogenic risk of molybdenum trioxide to the California public.

5. EPIDEMIOLOGICAL DATA

Droste et al. (1999).

The one epidemiological study claiming to be a positive occupational study of lung cancer (Droste et al., 1999) is based upon an examination of many mixed exposures to various substances, not just to molybdenum trioxide exposure, and is considered to be a poorly conducted study. This study of Belgian male lung cancer patients claims to be the first (and only) study to show an association between occupational exposure to molybdenum (sic) and lung cancer. However, this study is highly methodologically flawed (e.g., exposure was assessed only by self-report and by a job-task exposure matrix) and does not allow any conclusions to be drawn about the potential carcinogenicity of pure molybdenum trioxide. The study involved a hospital-based, case-control investigation with 478 lung cancer cases and 536 controls recruited from 10 hospitals in the Antwerp region. The authors reported that job histories in the categories "manufacturing of transport equipment other than automobiles," "transport support services" and "manufacturing of metal goods" were significantly associated with lung cancer. When assessed by job-task exposure matrix (JTEM), exposure to molybdenum, mineral oils and chromium were significantly associated with lung cancer risk.

The first source of bias in the paper results from over-selection of the control group. In an effort to "minimize the chance of controls having diseases that may be related to the exposures under study," the authors drew controls primarily from cardiovascular surgery wards and excluded subjects with "any type of cancer or with any primary lung disease." The net effect would have been a systematic and significant reduction in the likelihood that any control would have had industrial exposures. This is because exposures to dust and a wide array of industrial chemicals predispose to respiratory irritation and primary lung diseases. It is a fundamental rule of control selection in a case-control investigation that the controls must have the same opportunity for exposure as do the cases. By excluding control subjects with any type of primary lung disease, the authors would have preferentially excluded controls with industrial exposures. If this actually occurred, one would expect the control group to consist of a higher proportion of better educated and wealthier individuals, and that is apparent from the paper. The proportion of controls in the "High" education group exceeds the proportion of cases in that group by 47%, while the proportion of controls in the "High" socioeconomic status group exceeds the proportion of cases in that group by 45%. Exposure to molybdenum may have occurred more frequently among cases because the authors systematically excluded a subset of controls with industrial exposures.

A second set of methodological issues stems from the authors' method of exposure assessment. They used a combined strategy of directly asking subjects whether they had been exposed to certain potentially carcinogenic substances and then entering the subjects' reported occupations into a job-exposure matrix. Subjects in job categories with potential carcinogenic exposures were asked additional questions regarding their work tasks in order to generate a job task exposure matrix. Exposures were coded dichotomously (ever or never) as well as by the cumulative duration of exposure years. Since no effort was made to characterize or measure probable dose levels of exposure in the workplace, subjects with trivial exposures over a certain period of years would have been coded identically to individuals with heavy exposures over that same period of years.

In addition, there was apparently no differentiation of dose among various agents for subjects in job or task categories associated with exposure to multiple agents. For example, if a job task entailed heavy exposure to polycyclic aromatic hydrocarbons (PAHs) and asbestos, moderate exposure to arsenic, chromium and nickel, and low-level exposure to molybdenum, the exposure would have been encoded equally for all six. Of the eight job categories considered as entailing exposure to molybdenum, all eight entailed exposure to nickel, seven entailed exposure to arsenic, six entailed exposure to PAHs and six entailed exposure to asbestos. Given the wealth of data implicating those five substances as human lung carcinogens, the implication of molybdenum as a lung carcinogen is likely due to the confounding effect of those other known carcinogenic agents within an exposure matrix lacking dose information. Had the authors' analyses controlled for established industrial lung carcinogens, the molybdenum effect would likely have disappeared. Consequently, this study is not considered to provide any relationship between exposure to molybdenum and cancer.

Huvinen et al. (1996, 2002).

The only high-quality epidemiology study available that specifically addresses workers potentially exposed to molybdenum is a long-term study of ferrochromium and stainless steel manufacturers (Huvinen et al., 1996, 2002). The aim of this study, conducted in Finland, was to determine whether occupational exposure to chromite, trivalent chromium (Cr+3) or hexavalent chromium (Cr+6) caused respiratory diseases, an excess of respiratory symptoms, a decrease in pulmonary function or signs of pneumoconiosis among workers in stainless steel production. Altogether, 203 exposed workers and 81 referents with an average employment of 23 years were investigated on two occasions (in 1993 and 1998). Exposure to total dust and to different chromium species, as well as to other alloying metals (nickel and molybdenum) were monitored regularly and studied separately. The authors reported median air exposure concentrations in the steel melting shop for molybdenum of only 0.0003 mg/m^3 and 0.0006 mg/m^3 for personal and stationary samples, respectively, an exposure termed "low" by the authors.

The final conclusion of this study was that long term worker exposures (average 23 years) in modern ferrochromium and stainless steel production with low exposures to dusts and fumes containing chromium compounds, nickel and molybdenum did not lead to respiratory system changes detectable by reported symptoms, lung function tests or radiography.

6. GENOTOXICITY DATA

Molybdenum trioxide is non-genotoxic in the NTP assays and also in three assays conducted for IMOA by the Central Toxicology Laboratory, UK (CTL, 2004, 2005; attached). Some studies cited by OEHHA that purport to demonstrate positive genotoxicity effects are either studies of molybdenum compounds other than molybdenum trioxide or are deficient because of methodological flaws, particularly when the addition of molybdenum trioxide is known to reduce the pH of the assay systems' culture media and give false-positive effects due to the lowered pH.

NTP (1997).

The 1997 NTP Technical Report included a set of *in vitro* genetic toxicity tests on molybdenum trioxide in five strains of *Salmonella typhimurium* and cytogenetics tests for

chromosomal aberrations and sister chromatid exchanges in cultured Chinese hamster ovary cells. Concentrations of molybdenum trioxide used were 10 - 10,000 µg per plate and all tests were conducted in both the absence and the presence of induced hamster or rat liver S9. Pure molybdenum trioxide did not induce mutations in any of the *S. typhimurium* strains tested (TA97, TA98, TA100, TA1535 and TA 1537) with or without induced hamster or rat liver S9. Negative results were also obtained with molybdenum trioxide in the cytogenetics tests in cultured Chinese hamster ovary cells, and there was no induction of chromosomal aberrations or sister chromatid exchanges with or without S9. The NTP Technical Report concluded that "Molybdenum trioxide was not mutagenic in any of five strains of *Salmonella typhimurium*, and it did not induce sister chromatid exchanges or chromosomal aberrations in cultured Chinese hamster ovary cells *in vitro*."

Kerckaert et al., 1996; Gibson et al., 1997.

Positive results for *in vitro* micronucleus and cell transformation assays in Syrian hamster embryo (SHE) cells have been reported for many chemicals, including several metal compounds (Kerckaert et al., 1996; Gibson et al., 1997). Kerckaert et al. (1996) tested five metal chemicals in their SHE cell transformation assay, including molybdenum trioxide, cobalt sulfate hydrate, gallium arsenide, nickel (II) sulfate heptahydrate and vanadium pentoxide. The cobalt, gallium and vanadium compounds yielded significant morphological transformations at multiple doses of less than 1 µg/mL, the nickel sulfate required a dose of 5 µg/mL, but molybdenum trioxide required a dose of at least 75 µg/mL to yield significant morphological transformations, making it the weakest potency chemical among these other metals.

Gibson et al. (1997), on the other hand, tested 16 organic chemicals and metal compounds being tested at the time in NTP carcinogenicity studies in their *in vitro* SHE cell micronucleus assay. The main purpose of their study was to examine the overall concordance between induction of SHE cell micronuclei and other reports on transformation of SHE cells. They reported that molybdenum trioxide tested positive in their assay.

However, it is very likely that the results of these two studies of molybdenum trioxide are considered to be attributable to a significant reduction in the pH of the incubation media that is known to occur as a result of solubilization of molybdenum trioxide. There was evidence that dissolution of the molybdenum trioxide was associated with a significant reduction of pH in both water and in the incubation media used in these tests. It is well acknowledged that a reduction in pH in *in vitro* mammalian cell assays can result in false positive results. A report from the International Commission for the Protection Against Environmental Mutagens and Carcinogens (ICPEMC) recommended that "…positive results associated with pH shifts in the test system of greater than 1 unit should be viewed with caution and confirmed in experiments conducted at neutral pH" (Scott et al., 1991).

Titenko-Holland et al. (1998).

This study reported data from three assays: (1) an *in vitro* micronucleus assay of two molybdenum salts, ammonium molybdate and sodium molybdate, in human lymphocytes; (2) an *in vivo* mouse micronucleus assay of sodium molybdate in mouse bone marrow; and (3) a preliminary investigation using the *in vivo* mouse dominant lethal assay of sodium molybdate. The chemical formulas of ammonium molybdate and sodium molybdate differ significantly from molybdenum trioxide [MoO_3]:

$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ $Na_2MoO_4 \cdot H_2O$

The authors summarized their results as yielding "moderately positive results at relatively high doses in three experimental systems." However, IMOA considers that there are substantial weaknesses and flaws in the conduct of these studies. In addition, such studies should be conducted with molybdenum trioxide, since molybdenum trioxide's solubility characteristics are considerably different from other molybdate salts, and the products of reaction with biological fluids *in vivo* are unknown. Consequently, the studies reported by Titenko-Holland et al. (1998) provide no evidence for any *in vitro* or *in vivo* genotoxicity of molybdenum trioxide itself.

Central Toxicology Laboratory (2004, 2005).

In unpublished studies conducted by the Central Toxicology Laboratory, UK (CTL, 2004, 2005, attached), the IMOA commissioned studies on undensified sublimed pure molybdenum trioxide (Form A). This substance was tested in four strains of *S. typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and in *Escherichia coli* WP2P uvrA, in accordance with OECD Test Guideline 471. In order to evaluate the substance's clastogenic and aneugenic potential, it was also tested in an *in vitro* micronucleus assay using human lymphocytes in which the pH of the medium was adjusted to maintain the normal pH of the assay. In both assays, the compound was tested over a range of concentrations, both in the presence and absence of an induced rat liver-derived metabolic system (S9-mix). The bacterial tests were conducted in duplicate and the micronucleus test in triplicate. It was concluded that, under the conditions of the assays in bacteria, the "Form A" test substance, at concentrations from 100 - 5000 µg per plate, gave a non-mutagenic response in the tested strains of *S. typhimurium* and *E. coli* in both the presence and absence of metabolic activation, while positive control substances gave the expected responses. Furthermore, in the micronucleus assay, cytotoxicity was assessed by the use of binucleate index and genotoxicity was assessed by the incidence of micronucleated binucleate cells. "Form A" molybdenum trioxide also proved negative in this assay.

Genotoxicity Summary and Conclusions.

Pure molybdenum trioxide was found not to have any genotoxic activity in a series of well-conducted *in vitro* studies by both NTP and CTL. The positive *in vitro* micronucleus and cell transformation assays in SHE cells reported for molybdenum trioxide by Kerckaert et al. (1996) and Gibson et al. (1997) are considered to be flawed as evaluations of molybdenum trioxide, because there were artifacts arising from the reduced pH of the culture medium following dissolution of the molybdenum trioxide. It is concluded, therefore, that pure molybdenum trioxide tested at the proper pH is not genotoxic in these *in vitro* or *in vivo* assays, and that the positive assay results using ammonium molybdate and sodium molybdate should not be used to assess the genotoxicity of molybdenum trioxide.

7. HUMAN AND PLANT ESSENTIALITY OF MOLYBDENUM

Molybdenum is well known to be an essential trace mineral for humans, animals and plants (Food and Nutrition Board, FNB, 2001; Turnlund et al., 1995) involving several enzymes important to metabolism: mammalian xanthine oxidase/xanthine dehydrogenase, aldehyde

oxidase, sulfite oxidase, formate dehydrogenase, nitrate reductase and nitrogenase. It is also essential for plant production, even though present in plant tissue at a level much lower (0.5 ppm dry matter basis) than the critical levels for other essential elements. Molybdenum is needed for at least three human enzymes: (1) sulfite oxidase catalyses the oxidation of sulfite to sulfate, necessary for metabolism of sulfur amino acids, and sulfite oxidase deficiency or absence leads to neurological symptoms and early death; (2) xanthine oxidase catalyses oxidative hydroxylation of purines and pyridines including conversion of hypoxanthine to xanthine and xanthine to uric acid; and (3) aldehyde oxidase oxidizes purines, pyrimidines, pteridines and is involved in nicotinic acid metabolism. Low dietary molybdenum leads to low urinary and serum uric acid concentrations and excessive xanthine excretion.

The **Recommended Dietary Allowance (RDA)** for molybdenum for adult men and women is 45 µg/day. The average dietary intake of molybdenum (determined by the FNB) by adult men and women is 109 and 79 µg/day, respectively, and the median intake from supplements (determined by the Third National Health and Examination Survey) is about 23 and 24 µg/day for men and women who took supplements, respectively. It is known that the molybdenum content of plant foods varies depending upon the soil content in which they are grown, with legumes being the major contributors of dietary molybdenum, as well as grain products and nuts. Animal products, fruits and many vegetables are generally low in molybdenum. In addition, dietary supplements contain molybdenum in the form of added sodium molybdate, but molybdenum trioxide is not used in vitamin/mineral supplements.

Molybdenum also has several essential functions in plant growth and is required in a constant and continuous supply for normal assimilation of nitrogen. In this regard, it is a component of the enzyme nitrogenase, which is required in nitrogen fixation; legumes fix nitrogen, require more of it than cereals and thus are more sensitive to low molybdenum levels in soil. Sodium molybdate and ammonium molybdate are the molybdenum fertilizer materials most commonly used.

CONCLUSION

In conclusion, molybdenum trioxide should be given a Low Priority.

Sincerely yours,

Sandra Carey
HSE Executive
International Molybdenum Association
sandracarey@imoa.info
Tel: +44 (0) 7778 813721

James R. Coughlin, Ph.D.
President, Coughlin & Associates
27881 La Paz Rd., Suite G, PMB 213
Laguna Niguel, CA 92677
jrcoughlin@cox.net
Tel: 949-916-6217

[Approved but not signed]

F. Jay Murray, Ph.D.
President, Murray & Associates
5529 Perugia Circle
San Jose, CA 95138
jmurray2@sbcglobal.net
Tel: 408-239-0669

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APPENDIX I: Chemistry of Molybdenum Trioxide and Chemical Analysis of Molybdenum Compounds.

Physical Form of Molybdenum Trioxide (MoO_3) Tested in the NTP Bioassay.

Molybdenum trioxide is commercially produced in three forms, only one of which **("Form A" bolded in the following table)** was tested in the NTP 2-year chronic carcinogenicity inhalation bioassay.

	Form A **NTP Studies** **2-year inhalation**	*Form B* NTP did not study	*Form C* NTP 14-day & 13-week studies
Material description Mo trioxide	**Undensified sublimed pure**	Densified sublimed pure	Chemically produced pure
CAS No.	**1313-27-5**	1313-27-5	1313-27-5
Crystal morphology	**Acicular (needle-shaped)**	Irregular	Orthorhombic
Purity	**99.9%**	99.9%	99.9%
Solubility in water (20°C)	**1.40 g/L**	1.33 g/L	1.10 g/L
Malvern particle size (Vol Mean Diameter)	**39 µm**	262 µm	185 µm

As seen in the above table, the sublimed, undensified molybdenum trioxide (Form A) was the product that was provided to NTP for testing. However, there are two very important considerations that need to be noted regarding this product and the question of potential exposure of California workers or the public. First, as noted above, this product is not generally sold commercially in any significant quantities, and information from our member companies shows that none of this product is sold into California. Secondly, and more importantly, the NTP did not test this "Form A" product directly. Prior to exposing the rats and mice, the undensified molybdenum trioxide was micronized in a Trost air-impact mill to average particle sizes ranging from 1.3µm for the 10 mg/m^3 study to 1.5 µm for the 100 mg/m^3 mice exposure study, and for the rat study, average particle sizes ranging from 1.5 µm for the 10 mg/m^3 test series to 1.7 µm for the 100 mg/m^3 study. This significant reduction in particle size performed by NTP resulted in essentially all of the molybdenum trioxide being available to the lower lung area, which is over 600 times greater exposure to the lower lung than if the actual, undensified molybdenum trioxide "Form A" itself had been used in the bioassay. The end result is that the product tested by NTP is not, nor will ever be, shipped into California or contained in industrial products used in the State.

Chemical Analysis of Molybdenum Compounds.

Any attempts to measure actual molybdenum trioxide exposure concentrations in the environment, workplace, soil or foods (if present at all) will result in the measurement of only the molybdenum element, thus making exposure and speciation determinations of the

molybdenum trioxide molecule nearly impossible under Proposition 65. The element molybdenum (Mo) can be analyzed by several methods, including Inductively Coupled Plasma-Atomic Emission Spectrometry(ICP-AES), Neutron Activation Analysis and Atomic Absorption and Emission Spectroscopy (USGS website). However, these methods simultaneously analyze at least 10 - 40 other metals and metalloids, thus making exact chemical speciation of various molybdenum compounds an impossible analytical challenge. Consequently, all molybdenum compounds, including molybdenum trioxide and even the forms that occur naturally in foods, can only be reported analytically as elemental molybdenum concentrations.

APPENDIX II: Background on Statistical and Biological Significance of NTP Cancer Bioassay Findings.

Dr. Joseph K. Haseman was the NIEHS/NTP Chief of the Biostatistics Branch and Director of Statistical Consulting during his 33-year career there (retired in 2004), and he was primarily responsible for the experimental design and data analysis of the NTP rodent carcinogenicity program. Many of his papers concerned the statistical design and interpretation of NTP cancer bioassay results, which provide an understanding of how NTP uses statistically significant findings in interpreting its cancer bioassays.

Two early papers by Haseman (1983, 1984) formed the statistical basis for the currently conducted NTP bioassay program. Haseman pointed out that NTP believes that no rigid statistical decision rule should be the sole basis for the ultimate decision regarding a chemical's carcinogenicity. From his review of long-term bioassay studies completed at that time, Haseman (1983) described a statistical decision rule that closely approximates the scientific judgment process used to evaluate these studies:

> "Declare a compound carcinogenic if any common tumor showed a significant ($P < 0.01$) high-dose effect or if a $P < 0.05$ high-dose effect occurred for an uncommon tumor."

Said another way, Haseman's (1984) decision rule was described as follows:

> "…regard as carcinogenic any chemical that produces a high-dose increase in a common tumor that is statistically significant at the 0.01 level or a high-dose increase in an uncommon tumor that is statistically significant at the 0.05 level."

In essence, the key points made by Haseman were to be aware that it is not appropriate to blindly regard every $P < 0.05$ statistically positive finding as a biological positive, and that when a common tumor is being evaluated, it must reach the $P < 0.01$ level if it is to have any potential biological relevance. Haseman urged that other non-statistical factors must be considered before a final judgment is made regarding the carcinogenicity of a chemical. This statistical thinking is what is still in place in the interpretation of the modern NTP bioassay as well, i.e., the final interpretation of the data should be based on biological judgment rather than on the rigid application of statistical decision rules.

Haseman and Elwell (1996) published a major paper on the evaluation of false positive (i.e., tumor increases due to random variability that are incorrectly judged to be chemically-related) and false negative (i.e., real chemically-related effects dismissed as random variability) bioassay results. In fact, this paper was published shortly before NTP completed the Technical Report on molybdenum trioxide. The authors stated that it was well recognized that a decision procedure that routinely considers all statistically significant tumor increases to be biologically meaningful will have an unacceptably high false positive rate. In addition, they noted that because of the large number of potential target sites evaluated in a typical rodent cancer bioassay, statistically significant ($P < 0.05$) chemically-related tumor increases may arise by chance. They also listed in their publication several reasons why the NTP has historically <u>discounted</u> some statistically significant tumor increases seen in the rodent bioassays. The most common reasons included:

"(1) the tumor increase was not dose-related (e.g., a significant increase was observed at a low dose but was not supported by an increase at other dose levels),

(2) the tumor increase, while statistically significant, was only marginally so and involved a high spontaneous incidence tumor,

(3) the concurrent control tumor response was abnormally low, and/or

(4) the elevated tumor response in the dosed group fell within the range of values considered normal for controls of that sex and species."

The authors also defined in this paper the distinction between "common" tumors and "uncommon" (or rare) tumors occurring in rodents. "Common" tumors were defined as those tumor sites historically demonstrating a spontaneous rate greater than 2.0%, while the "uncommon" tumors occur at less than a 2.0% rate. They concluded that their analysis reflected "…the reality that common tumors are more likely to produce false positive outcomes than are uncommon tumors."

In the evaluation of laboratory animal carcinogenicity studies, Haseman (1995) had earlier pointed out that while the statistical significance of an observed tumor increase is important, "…the final interpretation of rodent carcinogenicity studies should not be based on rigid statistical decision rules, but rather on the exercise of informed scientific judgment." Additional factors cited by Haseman (1995) that should be used in judging the biological relevance of the findings included:

"(1) whether the effect was dose-related,

(2) whether the tumor increase was supported by an increase in related preneoplastic lesions,

(3) whether the effect was observed in other sex-species groups,

(4) whether the effect occurred in a suspected target organ, and

(5) the historical control rate of the tumor in question."

History of Chemically Induced Lung Lesions in NTP Bioassays.

NTP researchers recently published a comprehensive review and evaluation of all the lung tumor findings in 545 peer-reviewed NTP studies published to date (Dixon et al., 2008). They reported that the lung is the second most common target site (liver is the first) of neoplasia of the chemicals tested, with 64 chemicals in 66 reports producing significant neoplasias in the lungs of rats and/or mice (defined as "clear," "positive" or "some" evidence). Molybdenum trioxide was included in their analysis. Of the studies associated with lung tumor induction, approximately 35% were inhalation and 35% were gavage studies, with dosed-feed, dosed-water, topical, intraperitoneal or *in utero* routes of administration accounting for 18%, 6%, 3%, 1%, and 1% of the studies, respectively. The most commonly induced lung tumors were alveolar/bronchiolar (A/B) adenoma and/or carcinoma for both species, while the most frequently observed nonneoplastic lesions included hyperplasia of the

alveolar epithelium and inflammation in both species. The liver was the most common primary site of origin of metastatic lesions to the lungs of mice.

Sublimed Undensified Molybdenum Trioxide: Bacterial Mutation Assay in S. Typhimurium & E. Coli

Study No. CTL/YV6553 conducted in 2003

by

Central Toxicology Laboratory
Alderley Park, Macclesfield,
Cheshire, United Kingdom

Prepared for:

International Molybdenum Association (**IMOA**)
4 Heathfield Terrace, Chiswick
London, W4 4JE

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CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK

CTL/YV6553/REGULATORY/REPORT

SUBLIMED UNDENSIFIED PURE MOLYBDENUM TRIOXIDE: BACTERIAL MUTATION ASSAY IN *S.TYPHIMURIUM* AND *E.COLI*

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CTL/YV6553/REGULATORY/REPORT

SUBLIMED UNDENSIFIED PURE MOLYBDENUM TRIOXIDE: BACTERIAL MUTATION ASSAY IN *S.TYPHIMURIUM* AND *E.COLI*

STUDY DETAILS

Sponsor:	International Molybdenum Association 2 Baron's Gate 33 Rothschild Road London W4 5HT
Sponsor Reference:	C03162r1
CTL Test Substance Reference Number:	Y12618/001
CTL Study Number:	YV6553
Document Number:	CTL/YV6553/REG/REPT

AUTHOR

Eryl Jones

DATE OF ISSUE

1 April 2004

STATEMENT OF DATA CONFIDENTIALITY CLAIM

THIS DOCUMENT CONTAINS INFORMATION CONFIDENTIAL AND TRADE SECRET TO INTERNATIONAL MOLYBDENUM ASSOCIATION.

It should not be disclosed in any form to an outside party, nor should information contained herein be used by a registration authority to support registration of this product or any other product without the written permission of International Molybdenum Association.

STATEMENT OF GLP COMPLIANCE AND AUTHENTICATION

I, the undersigned, declare that the objectives laid down in the protocol were achieved and that the data generated are valid. The report fully and accurately reflects the procedures used and the raw data generated in the above study.

The study (YV6553) was conducted in compliance with the UK Principles of Good Laboratory Practice (The United Kingdom GLP Regulations 1999, Statutory Instrument No. 3106) except for the deviation listed below. These Principles are in accordance with the OECD Principles of Good Laboratory Practice, revised 1997 (ENV/MC/CHEM(98)17).

The following GLP deviation is considered not to affect the integrity of the study or the validity of the conclusions drawn:

(i) the stability, homogeneity and achieved concentration of the test substance in the vehicle used were not determined by analysis.

Eryl Jones Study Director	.....	1 April 2004 Date

This page
may be required
by some
regulatory authorities.

QUALITY ASSURANCE STATEMENT

In accordance with CTL policy and QA procedures for Good Laboratory Practice, this report has been audited and the conduct of this study has been inspected as follows:

Date	Audit/Inspection	Date of QA Report
03 Nov 2003	Draft report	03 Nov 2003
01 Apr 2004	Final report review	01 Apr 2004

In addition, inspections associated with this type of study were made as follows:

08 Jul 2003	Dose preparation	08 Jul 2003
08 Jul 2003	Dilutions, plating	08 Jul 2003
09 Jul 2003	Protocol	09 Jul 2003
11 Jul 2003	Counting	11 Jul 2003

Facilities and process based procedures associated with this type of study were inspected in accordance with QA Standard Operating Procedures.

So far as can be reasonably established, the methods described and the results given in the final report accurately reflect the raw data produced during the study, YV6553.

C Rooney ..... 1 April 2004

(CTL Quality Assurance Unit)

STUDY CONTRIBUTORS

The following contributed to this report in the capacities indicated:

Name	Title
Eryl Jones	Study Director CTL
B M Elliott	Study Reviewer

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This page is provided for the Regulatory Authority Reviewer's notes.

1. SUMMARY

1.1 Study design

Sublimed undensified pure molybdenum trioxide (CAS No. 1313-27-5) was evaluated in a bacterial mutagenicity assay (based on Maron and Ames (1983)) over a range of concentrations using four strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and one strain of *Escherichia coli* (WP2P *uvrA*) in the presence and absence of a rat liver - derived metabolic activation system (S9-mix).

1.2 Results

In two separate assays with each tester strain, the test substance did not induce any significant, reproducible increases in the observed number of revertant colonies either in the presence or absence of metabolic activation

The sensitivity of the test system, and the metabolic activity of the S9-mix, were clearly demonstrated by the increases in the numbers of revertant colonies induced by positive control substances.

1.3 Conclusion

It is concluded that, under the conditions of this assay, sublimed undensified pure molybdenum trioxide gave a negative, i.e. non-mutagenic response in *S.typhimurium* strains TA1535, TA1537, TA98 and TA100 and *E.coli* strain WP2P *uvr*A in both the presence and absence of metabolic activation.

2. INTRODUCTION

2.1 Purpose

The purpose of this study was to investigate whether the test substance induced gene mutation in certain strains of the bacteria *Salmonella typhimurium* and *Escherichia coli*.

2.2 Regulatory guidelines

The study was conducted according to the following Regulatory Guidelines:

a) Annex V to Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances, published in the 26th Adaptation, Commission Directive 2000/32/EEC of 19 May 2000, OJ L136 8.6.2000 (title amended in the 28th Adaptation, Commission Directive 2001/59/EEC of 6 August 2001, OJ L225 21.8.2001). B.13/14, Mutagenicity - reverse mutation test using bacteria.

b) ICH Harmonised Tripartite Guidelines S2A: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (adopted at Step 4 of the ICH process 19 July 1995) and S2B: Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals (adopted at Step 4 of the ICH process 16 July 1997).

c) OECD guideline reference 471 (1997). Bacterial Reverse Mutation Assay.

d) USEPA Health Effects Test Guideline OPPTS 870.5100 (1998). Bacterial Reverse Mutation Test.

2.3 Justification for test system selection

The bacterial mutation assay was used as it is a well-established assay, designed to detect reversion to amino acid independence (his^- to his^+ for *S.typhimurium*, trp^- to trp^+ for *E.coli* strains) induced by chemicals which cause base changes or frameshift mutations in the genome of these organisms. The use of strains TA1535, TA1537, TA98, TA100 and WP2P *uvrA* is in accordance with the current scientific recommendations for the conduct of this assay.

2.4 Study dates

The study was initiated on 22 August 2003. The experimental phase was started on 26 August 2003 and was completed on 10 October 2003.

2.5 Data storage

An original report, the study protocol and all raw data pertaining to this study will be retained in the CTL Archives, Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK for a minimum of 5 years from the date of issue of the final report. At the end of this period the Sponsor will be contacted regarding the further retention, return to the Sponsor or destruction of the archived materials.

3. TEST AND CONTROL SUBSTANCES

3.1 Test substance

Name:	Sublimed undensified pure molybdenum trioxide
CAS number	1313-27-5
Source:	IMOA
Colour:	Pale green
Physical state:	Powder
Batch reference:	Lot 1MS
CTL test substance reference number:	Y12618/001
Purity:	100%
Storage conditions:	Cool, dark well ventilated area

From the information supplied by the Sponsor, the test substance is stable for a period in excess of one year. A certificate of analysis (dated 14/07/03) is retained in the CTL Archives. The characterisation of the test substance is the responsibility of the Sponsor. In all cases where the concentration of test substance is quoted, the concentration refers to the concentration of test substance not corrected for purity.

3.2 Control substances

The negative control substance and solvent for the test sample was dried dimethylsulphoxide (CTL test substance reference number Y00876/011). The positive control substances are detailed in the following table:

Chemical	Supplier	CTL Ref	Solvent
Acridine Mutagen ICR191	Sigma	Y03243/003	DMSO
2-Aminoanthracene (2AA)	Sigma	Y01142/006	DMSO
Benzo [*a*] pyrene (BP)	Lancaster Synthesis	Y00111/005	DMSO
Daunomycin HCl (DR)	Sigma	Y01165/002	DMSO
N-Ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	Sigma	Y02125/001	DMSO
Sodium Azide (NaZ)	Sigma	Y06019/001	H_2O

4. EXPERIMENTAL PROCEDURE

4.1 Dosing preparations

Individual stock solutions/suspensions of the test substance were prepared for each experiment at an appropriate concentration in dried dimethylsulphoxide and serial dilutions were carried out as required in each case.

The positive control substances were prepared as solutions in the solvents detailed in the table above.

All test and positive control substance dosing preparations were prepared as close to the time of culture treatment as possible and were dosed at a dosing volume of 100µl/plate.

4.2 Analysis of dosing preparations

In view of the short-term nature of the studies of this type, no analyses of stability, homogeneity or achieved concentrations were carried out on the preparations of the positive control or test substances either prior to or after addition to the bacteria.

4.3 Experimental design

The principle of the assay was as described by Maron and Ames (1983) and the procedure employed followed the Ames protocol modified in accordance with the procedures outlined in OECD guideline 471.

The four *Salmonella typhimurium* tester strains (TA1535, TA1537, TA98 and TA100) and the one *Escherichia coli* strain (WP2P *uvrA* [WP2 *uvrA* (pKM101)] are fully described in Ames *et al* (1975) and references therein, and in Venitt and Crofton-Sleigh (1979).

Sublimed undensified pure molybdenum trioxide was initially assayed using the standard plate incorporation protocol over a dose range of 5000 to 100µg per plate, both in the presence and absence of S9-mix prepared from phenobarbital/β-naphthoflavone-induced Sprague-Dawley (SD) rats (see Section 4.5). The test substance was subsequently re-tested in all five strains over the same dose range, In the repeat experiment, in the presence of S9-mix, a pre-incubation procedure was used as recommended by the regulatory guidelines. The incubation period for each experiment was 3 days (at 37°C).

For each experiment, positive control substances were tested to validate the bacterial strains and to confirm the activity of the S9-mix used.

The experimental design for each strain was as follows:

a) Sublimed undensified pure molybdenum trioxide	(at least) 6 concentrations per strain as shown in Tables 1-5 3 plates per concentration
b) Solvent Control: dried dimethylsulphoxide (100µl)	5 plates
c) Positive Controls +S9 : 2AA (all Salmonella strains) BP (WP2P *uvrA*)	1 concentration per strain as shown in Tables 1-5
-S9 : DR (TA98) ENNG (WP2P *uvrA*) ICR 191 (TA1537) NaZ (TA1535 and TA100)	3 plates per concentration

4.4 Bacterial cultures

Stock cultures of each strain were prepared and maintained in liquid nitrogen as described by Maron and Ames (1983). The routine source of inocula for overnight cultures was one set of frozen stocks, used and discarded on an approximately weekly basis. The overnight culture from each new frozen culture was screened for the deep-rough character, DNA repair deficiency and Ampicillin resistance as previously described (Maron and Ames (1983) with one modification:-

> The presence of the *uvrB* deletion (*S.typhimurium*) and the *uvrA* mutation (*E.coli*) was confirmed by testing the sensitivity of each culture to mitomycin C (10µl of a 10µg/ml solution) in the same manner as sensitivity to crystal violet was tested. Damage to DNA caused by mitomycin C is repaired in normal bacteria by the *uvr* excision repair pathway, and is thus toxic to strains deficient at either the *uvrA* or *uvrB* loci.

When fresh frozen stocks were prepared, the strains were tested for amino acid requirement and for reversion properties using diagnostic mutagens as described by Ames *et al* (1975) and Maron and Ames (1983), except that the mutagens were incorporated in the top agar layer as in a standard experiment (see Section 4.6.1), rather than spot tested as described by Ames *et al*.

4.5 Metabolic activation system

This work was conducted with and without S9-mix incorporated in the top-agar. The S9-mix was prepared as required (on each day of experimentation) as follows:

	Volume per 30ml S9-mix
S9 fraction	3ml
Sucrose-Tris-EDTA buffer (S9 buffer)	7ml
Cofactor solution	20ml

In tests without metabolic activation, the S9 fraction and cofactor solution were replaced by an equivalent volume of S9 buffer. Both the S9-mix and the S9 buffer were kept on ice until used.

S9 was purchased from MolTox Inc., and was prepared from male Sprague Dawley rats, dosed once daily (by oral gavage) for 3 days with a combined phenobarbital (80mg/kg bodyweight) and β-naphthoflavone (100mg/kg) corn oil solution. The treated animals were sacrificed on the day following the third dose. A 25% w/v homogenate (the S9 fraction) was prepared according to the method given in Callander *et al* (1995).

The cofactor solution was prepared as a single stock solution of Na_2HP0_4, KCl, glucose-6-phosphate, NADP (Na salt) and $MgCl_2$ (150:49.5:7.5:6:12 mM) in sterile deionised water, and adjusted to a final pH of 7.4.

4.6 Methodology

0.1ml aliquots of an overnight culture (10-12 hours) of each bacterial strain were dispensed into the required number of appropriate containers (bijoux), and stored at room temperature until required.

Top agar consisting of 0.6% w/v agar and 0.5% w/v sodium chloride in deionised water was melted by brief autoclaving and stored at approximately 50°C until required.

Prior to testing the molten top agar was prepared by adding sterile 0.5mM histidine/0.5mM biotin stock solution (10ml solution:100ml agar) for Salmonella work, and by adding sterile tryptophan solution (10ml 0.5mM stock:100ml agar) for *E.coli* work. Separate agars were prepared for each species.

4.6.1 Plate incorporation protocol

0.5ml S9-mix (or S9 buffer) was then added to the number of aliquots of one strain required for one concentration, followed by 0.1ml of the appropriate concentration of test substance preparation. Finally, 2.0ml top agar was then added to each aliquot, and the resulting mixture poured rapidly onto the surface of a prepared pre-labelled Vogel Bonner plate (9cm diameter vented Petri-dish prepared with 25ml Vogel Bonner minimal medium and containing 1.5% w/v agar and 2% w/v glucose) and allowed to gel. Plates were then incubated inverted at 37°C for 3 days in the dark.

At the end of the incubation period the plates were examined for the lack of microbial contamination and evidence that the test was valid: i.e. there was a background lawn on the solvent control plates and on the plates for (at least) the lower concentrations of test substance, and that the positive controls had responded as expected. All plates were counted using an automated colony counter adjusted appropriately to permit the optimal counting of mutant colonies. Plates that were obviously contaminated were recorded as such without counting.

4.6.2 Pre-incubation protocol

The assay procedure was as for the plate-incorporation protocol described above, except that

a) each compound/solvent dose was added in 0.02ml volumes, with the total dose volume made up to 0.1ml with phosphate buffered saline;

b) before adding the top agar, each compound/strain group of bijoux was placed on an orbital shaker (at approximately 140rpm) for a pre-incubation period of 60 minutes at 37°C.

5. DATA EVALUATION

Test data from individual experiments are considered valid if:

a) the concurrent solvent control data are acceptable;

b) the positive control data show acceptable increases;

Failure of one or more tester strain/S9 combinations does not invalidate the data for the remainder of a concurrent experiment.

A positive response in a (valid) individual experiment is achieved when one or both of the following criteria are met:

a) a significant, dose-related increase in the mean number of revertants is observed;

b) a two-fold or greater increase in the mean number of revertant colonies (over that observed for the concurrent solvent control plates) is observed at one or more concentrations

A negative result in a (valid) individual experiment is achieved when:

a) there is no significant dose-related increase in the mean number of revertant colonies per plate observed for the test substance; and

b) in the absence of any such dose response, no increase in colony numbers is observed (at any test concentration) which exceeds 2x the concurrent solvent control.

For a positive response in an individual experiment to be considered indicative of an unequivocal positive, i.e. mutagenic, result for that strain/S9 combination, then the observed effect(s) must be consistently reproducible.

All derived calculations (i.e. mean colony count/plate; standard deviation, etc.) shown in the results tables were carried out by computer. Counts from contaminated plates are not included in these calculations.

6. RESULTS

The numbers of revertant colonies per plate observed in the four *Salmonella typhimurium* tester strains (TA1535, TA1537, TA98 and TA100) and one *Escherichia coli* strain (WP2P *uvrA*), when exposed to sublimed undensified pure molybdenum trioxide and to the various positive and solvent control substances are shown in Tables 1-4. All derived values in the results tables are expressed, where appropriate, to one decimal place: values of 0.05+ have been rounded up to 0.1.

In two separate assays with each tester strain, the test substance did not induce any significant, reproducible increases in the observed number of revertant colonies either in the presence or absence of S9-mix (Tables 1-4).

Signs of toxicity of the test substance at the highest concentration tested (i.e. a sparseness of the background bacterial lawn) were observed occasionally with some strains in both the presence and absence of S9-mix.

Precipitation of the test substance in the presence of S9-mix was observed in the plate test at the highest concentration in TA100 , WP2P *uvrA* and also at the highest concentration in the pre-incubation experiment in all bacterial strains.

The positive controls for each experiment induced the expected responses (Tables 1-4), indicating the strains were responding satisfactorily in each case.

7. CONCLUSIONS

It is concluded that, under the conditions of this assay, sublimed undensified pure molybdenum trioxide gave a negative, i.e. non-mutagenic response in *S.typhimurium* strains TA1535, TA1537, TA98 and TA100 and *E.coli* strain WP2P *uvr*A in both the presence and absence of metabolic activation.

8. REFERENCES

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Maron D M and Ames B N (1983). Revised methods for the Salmonella mutagenicity test. Mutation Res **113**, 173-215.

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TABLE 1 - DATA FOR EXPERIMENTAL PHASE 1 (PLATE INCORPORATION: +S9-MIX)

Study Name: YV6553: Sublimed undensified pure molybdenum trioxide
Experiment: YV6553: Phase 1
Assay Conditions: Standard plate incorporation assay

Study Code: YV6553
Date Plated: 26/08/2003
Counted: 29/08/2003

With S9-mix

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	**Y12618/001**	5000µg	*12.7*	*7.6*	*0.1*	18 S P, 4 S P, 16 S P
		2500µg	*110.3*	*4.7*	*0.7*	114, 105, 112
		1000µg	*88.7*	*51.7*	*0.6*	116, 121, 29
		500µg	*135.7*	*18.6*	*0.9*	155, 118, 134
		200µg	*108.7*	*14.0*	*0.7*	108, 95, 123
		100µg	*140.3*	*26.1*	*0.9*	149, 111, 161
	DMSO		*153.8*	*18.1*		152, 163, 128, 149, 177
TA1535	**Y12618/001**	5000µg	*4.0*	*2.0*	*0.4*	6 S, 2 S, 4 S
		2500µg	*6.3*	*2.3*	*0.6*	9, 5, 5
		1000µg	*7.7*	*3.2*	*0.8*	10, 4, 9
		500µg	*8.0*	*3.6*	*0.8*	12, 5, 7
		200µg	*7.0*	*3.0*	*0.7*	10, 7, 4
		100µg	*9.0*	*2.0*	*0.9*	11, 9, 7
	DMSO		*10.0*	*2.0*		11, 12, 9, 11, 7
TA1537	**Y12618/001**	5000µg	*0.3*	*0.6*	*0.0*	0 S, 0 S, 1 S
		2500µg	*3.3*	*2.9*	*0.5*	5, 5, 0
		1000µg	*9.0*	*0.0*	*1.3*	9, 9, 9
		500µg	*6.3*	*2.3*	*0.9*	9, 5, 5
		200µg	*10.7*	*4.5*	*1.5*	15, 11, 6
		100µg	*11.7*	*6.0*	*1.7*	18, 6, 11
	DMSO		*7.0*	*2.0*		9, 5, 5, 9, 7
TA98	**Y12618/001**	5000µg	*19.7*	*4.0*	*0.7*	22, 22, 15
		2500µg	*22.0*	*5.0*	*0.8*	22, 17, 27
		1000µg	*22.7*	*7.0*	*0.8*	22, 30, 16
		500µg	*29.3*	*2.5*	*1.0*	29, 27, 32
		200µg	*24.0*	*1.0*	*0.8*	24, 23, 25
		100µg	*35.3*	*6.7*	*1.2*	28, 41, 37
	DMSO		*29.0*	*8.8*		30, 22, 21, 29, 43
WP2 uvrA (pKM101)	**Y12618/001**	5000µg	*159.3*	*22.4*	*0.7*	135 P, 179 P, 164 P
		2500µg	*200.0*	*22.6*	*0.9*	206, 219, 175
		1000µg	*244.0*	*39.9*	*1.1*	198, 270, 264
		500µg	*229.0*	*34.5*	*1.1*	264, 228, 195
		200µg	*224.3*	*15.1*	*1.0*	207, 231, 235
		100µg	*248.3*	*2.5*	*1.2*	251, 246, 248
	DMSO		*215.8*	*13.6*		196, 231, 214, 226, 212
TA100	**2AA**	1µg	*442.0*	*53.4*	*2.9*	500, 395, 431
TA1535	**2AA**	2µg	*75.7*	*16.8*	*7.6*	95, 67, 65
TA1537	**2AA**	2µg	*76.0*	*8.7*	*10.9*	66, 82, 80
TA98	**2AA**	1µg	*537.0*	*156.6*	*18.5*	649, 604, 358
WP2 uvrA (pKM101)	**BP**	5µg	*1806.0*	*68.8*	*8.4*	1880, 1744, 1794

Key to Positive Controls

2AA	2-Aminoanthracene
BP	Benzo[a]pyrene

Key to Plate Postfix Codes

S	Sparse/Incomplete lawn
P	Precipitate

TABLE 2 - DATA FOR EXPERIMENTAL PHASE 1 (PLATE INCORPORATION: -S9-MIX)

Study Name: YV6553: Sublimed undensified pure molybdenum trioxide
Experiment: YV6553: Phase 1
Assay Conditions: Standard plate incorporation assay

Study Code: YV6553
Date Plated: 26/08/2003
Counted: 29/08/2003

Without S9-mix

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	**Y12618/001**	5000µg	*81.7*	*12.9*	*0.7*	78, 96, 71
		2500µg	*119.7*	*23.3*	*1.1*	136, 130, 93
		1000µg	*102.7*	*25.8*	*0.9*	124, 110, 74
		500µg	*92.0*	*8.5*	*0.8*	83, 100, 93
		200µg	*105.3*	*15.3*	*0.9*	88, 117, 111
		100µg	*95.3*	*6.4*	*0.9*	99, 99, 88
	DMSO		*111.4*	*11.3*		108, 113, 94, 119, 123
TA1535	**Y12618/001**	5000µg	*3.7*	*1.5*	*0.3*	4, 5, 2
		2500µg	*6.3*	*0.6*	*0.6*	6, 7, 6
		1000µg	*6.3*	*0.6*	*0.6*	6, 6, 7
		500µg	*8.7*	*4.5*	*0.8*	9, 4, 13
		200µg	*5.0*	*1.0*	*0.4*	6, 4, 5
		100µg	*6.3*	*3.2*	*0.6*	4, 5, 10
	DMSO		*11.2*	*4.9*		12, 6, 10, 19, 9
TA1537	**Y12618/001**	5000µg	*0.7*	*1.2*	*0.1*	2, 0, 0
		2500µg	*3.7*	*3.5*	*0.5*	7, 0, 4
		1000µg	*3.3*	*1.2*	*0.5*	4, 4, 2
		500µg	*3.3*	*2.3*	*0.5*	6, 2, 2
		200µg	*6.3*	*0.6*	*0.9*	7, 6, 6
		100µg	*5.3*	*1.2*	*0.8*	4, 6, 6
	DMSO		*7.0*	*2.0*		7, 5, 5, 9, 9
TA98	**Y12618/001**	5000µg	*19.3*	*3.5*	*1.1*	23, 19, 16
		2500µg	*18.3*	*3.1*	*1.1*	21, 15, 19
		1000µg	*19.7*	*4.2*	*1.2*	15, 23, 21
		500µg	*20.3*	*9.6*	*1.2*	29, 10, 22
		200µg	*19.3*	*8.1*	*1.1*	18, 12, 28
		100µg	*19.0*	*5.3*	*1.1*	21, 13, 23
	DMSO		*17.0*	*5.5*		11, 12, 23, 17, 22
WP2 uvrA (pKM101)	**Y12618/001**	5000µg	*143.7*	*5.5*	*1.0*	141, 140, 150
		2500µg	*144.7*	*33.6*	*1.0*	108, 174, 152
		1000µg	*154.0*	*32.9*	*1.0*	116, 174, 172
		500µg	*138.3*	*19.6*	*0.9*	133, 122, 160
		200µg	*133.3*	*27.6*	*0.9*	103, 140, 157
		100µg	*145.3*	*30.6*	*1.0*	110, 164, 162
	DMSO		*148.2*	*15.0*		145, 149, 149, 170, 128
TA100	**NaZ**	2µg	*854.0*	*157.0*	*7.7*	699, 1013, 850
TA1535	**NaZ**	2µg	*492.7*	*63.0*	*44.0*	420, 531, 527
TA1537	**ICR**	2µg	*191.0*	*24.4*	*27.3*	174, 219, 180
TA98	**DR**	1µg	*962.3*	*75.1*	*56.6*	1040, 890, 957
WP2 uvrA (pKM101)	**ENNG**	1µg	*341.7*	*33.6*	*2.3*	303, 364, 358

Key to Positive Controls

NaZ	Sodium Azide
ICR	Acridine Mutagen ICR191
DR	Daunomycin Hydrochloride
ENNG	N-Ethyl-N'-nitro-N-nitrosoguanidine

TABLE 3 - DATA FOR EXPERIMENTAL PHASE 2 (PRE-INCUBATION: +S9-MIX)

Study Name: YV6553: Sublimed undensified pure molybdenum trioxide
Experiment: YV6553 Phase 4
Assay Conditions: Standard pre-incubation assay

Study Code: YV6553
Date Plated: 07/10/2003
Counted: 10/10/2003

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	**Y12618/001**	5000µg	*93.7*	*11.1*	*0.6*	104 P, 95 P, 82 P
		2500µg	*120.0*	*24.5*	*0.8*	95, 121, 144
		1000µg	*169.7*	*23.2*	*1.1*	189, 176, 144
		500µg	*170.3*	*28.7*	*1.1*	193, 180, 138
		200µg	*188.3*	*23.6*	*1.2*	213, 186, 166
		100µg	*153.7*	*19.9*	*1.0*	131, 168, 162
	DMSO		*152.2*	*15.2*		164, 152, 142, 170, 133
TA1535	**Y12618/001**	5000µg	*8.0*	*3.6*	*0.7*	5 P, 12 P, 7 P
		2500µg	*8.7*	*2.9*	*0.7*	7, 12, 7
		1000µg	*10.3*	*1.5*	*0.9*	10, 12, 9
		500µg	*6.0*	*0.0*	*0.5*	6, 6, 6
		200µg	*11.3*	*4.7*	*1.0*	15, 13, 6
		100µg	*12.0*	*5.0*	*1.0*	7, 12, 17
	DMSO		*11.6*	*5.8*		9, 13, 21, 6, 9
TA1537	**Y12618/001**	5000µg	*0.0*	*0.0*	*0.0*	0 P, 0 P, 0 P
		2500µg	*1.0*	*1.0*	*0.1*	0, 2, 1
		1000µg	*2.3*	*1.5*	*0.3*	1, 4, 2
		500µg	*8.3*	*3.8*	*1.0*	10, 11, 4
		200µg	*4.0*	*3.0*	*0.5*	4, 7, 1
		100µg	*9.7*	*3.5*	*1.2*	13, 10, 6
	DMSO		*8.4*	*2.4*		7, 11, 11, 6, 7
TA98	**Y12618/001**	5000µg	*28.7*	*9.8*	*1.1*	40 P, 23 P, 23 P
		2500µg	*24.3*	*1.5*	*1.0*	24, 26, 23
		1000µg	*30.0*	*6.2*	*1.2*	32, 23, 35
		500µg	*33.7*	*7.1*	*1.3*	26, 35, 40
		200µg	*27.7*	*1.2*	*1.1*	27, 27, 29
		100µg	*39.3*	*8.4*	*1.6*	35, 34, 49
	DMSO		*25.2*	*4.7*		28, 21, 20, 31, 26
WP2 uvrA (pKM101)	**Y12618/001**	5000µg	*176.0*	*20.0*	*1.1*	154 P, 193 P, 181 P
		2500µg	*167.0*	*36.3*	*1.0*	136, 158, 207
		1000µg	*196.7*	*30.2*	*1.2*	211, 217, 162
		500µg	*185.7*	*5.7*	*1.1*	184, 192, 181
		200µg	*173.0*	*21.0*	*1.0*	158, 197, 164
		100µg	*192.0*	*3.0*	*1.2*	195, 189, 192
	DMSO		*166.8*	*22.4*		133, 157, 173, 189, 182
TA100	**2AA**	1µg	*893.0*	*258.0*	*5.9*	1153, 889, 637
TA1535	**2AA**	2µg	*222.0*	*29.5*	*19.1*	213, 255, 198
TA1537	**2AA**	2µg	*237.3*	*11.7*	*28.3*	242, 224, 246
TA98	**2AA**	1µg	*2056.7*	*422.5*	*81.6*	1572, 2251, 2347
WP2 uvrA (pKM101)	**BP**	5µg	*936.7*	*37.6*	*5.6*	894, 965, 951

2AA 2-Aminoanthracene
BP Benzo[a]pyrene

P Precipitate

TABLE 4 - DATA FOR EXPERIMENTAL PHASE 2 (PLATE INCORPORATION: -S9-MIX)

Study Name: YV6553: Sublimed undensified pure molybdenum trioxide
Experiment: YV6553: Phase 2
Assay Conditions: Standard plate incorporation assay

Study Code: YV6553
Date Plated: 05/09/2003
Counted: 08/09/2003

Without S9-mix

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	**Y12618/001**	5000µg	*32.0*	*5.6*	*0.2*	26 S, 33 S, 37 S
		2500µg	*77.0*	*2.0*	*0.6*	79, 77, 75
		1000µg	*103.3*	*14.3*	*0.8*	91, 100, 119
		500µg	*115.0*	*9.2*	*0.8*	105, 123, 117
		200µg	*133.7*	*9.7*	*1.0*	123, 142, 136
		100µg	*99.7*	*15.7*	*0.7*	82, 105, 112
	DMSO		*136.0*	*12.4*		134, 156, 124, 138, 128
TA1535	**Y12618/001**	5000µg	*3.3*	*1.2*	*0.5*	4, 4, 2
		2500µg	*4.0*	*2.0*	*0.6*	6, 2, 4
		1000µg	*2.7*	*1.2*	*0.4*	2, 2, 4
		500µg	*6.0*	*3.5*	*0.9*	4, 10, 4
		200µg	*9.0*	*3.0*	*1.4*	9, 6, 12
		100µg	*7.7*	*2.1*	*1.2*	6, 10, 7
	DMSO		*6.6*	*1.8*		9, 6, 7, 7, 4
TA1537	**Y12618/001**	5000µg	*1.0*	*1.0*	*0.1*	1, 2, 0
		2500µg	*2.0*	*1.7*	*0.3*	1, 4, 1
		1000µg	*2.3*	*1.5*	*0.3*	1, 2, 4
		500µg	*3.7*	*1.5*	*0.5*	4, 5, 2
		200µg	*1.7*	*2.1*	*0.2*	0, 4, 1
		100µg	*6.0*	*1.7*	*0.9*	7, 7, 4
	DMSO		*6.8*	*3.0*		6, 4, 4, 10, 10
TA98	**Y12618/001**	5000µg	*16.7*	*1.5*	*0.8*	15, 17, 18
		2500µg	*18.0*	*1.7*	*0.9*	16, 19, 19
		1000µg	*19.7*	*3.2*	*1.0*	16, 21, 22
		500µg	*13.0*	*5.3*	*0.7*	17, 15, 7
		200µg	*18.7*	*8.3*	*0.9*	12, 28, 16
		100µg	*15.3*	*6.0*	*0.8*	9, 21, 16
	DMSO		*19.8*	*7.2*		18, 12, 30, 24, 15
WP2 uvrA (pKM101) *	**Y12618/001**	5000µg	*154.0*	*3.6*	*1.2*	153, 151, 158
		2500µg	*129.3*	*20.5*	*1.0*	124, 152, 112
		1000µg	*122.7*	*14.0*	*0.9*	107, 134, 127
		500µg	*125.7*	*13.2*	*1.0*	114, 140, 123
		200µg	*114.3*	*16.2*	*0.9*	97, 117, 129
		100µg	*116.0*	*7.0*	*0.9*	108, 121, 119
	DMSO		*130.0*	*12.9*		114, 141, 139, 138, 118
TA100	**NaZ**	2µg	*1042.0*	*28.9*	*7.7*	1063, 1054, 1009
TA1535	**NaZ**	2µg	*660.0*	*52.3*	*100.0*	654, 611, 715
TA1537	**ICR**	2µg	*167.7*	*19.9*	*24.7*	190, 161, 152
TA98	**DR**	1µg	*990.3*	*191.5*	*50.0*	1204, 933, 834
WP2 uvrA (pKM101)	**ENNG** *	1µg	*272.0*	*22.3*	*2.1*	296, 252, 268

Key to Positive Controls

NaZ	Sodium Azide
ICR	Acridine Mutagen ICR191
DR	Daunomycin Hydrochloride
ENNG	N-Ethyl-N'-nitro-N-nitrosoguanidine

Key to Plate Postfix Codes

S	Sparse/Incomplete lawn

* Due to failure of the positive control in the initial experiment, data are from a separate experiment (poured: 15/09/2003,counted: 18/09/03)

APPENDIX 1 - HISTORICAL CONTROL DATA

	+S9 Plate Incorporation		+S9 Pre-Incubation		-S9	
TA 100	**Solvent Controls**	**1.0µg 2AA**	**Solvent Controls**	**1.0µg 2AA**	**Solvent Controls**	**2.0µg NaZ**
Mean ± SD	130 ± 38	1222 ± 647	132 ± 35	1563 ± 850	123 ± 36	914 ± 372
N	649	255	609	244	1228	488
Minimum	41	365	60	348	55	310
Maximum	245	3720	228	4430	232	2632
TA 1535	**Solvent Controls**	**2.0µg 2AA**	**Solvent Controls**	**2.0µg 2AA**	**Solvent Controls**	**2.0µg NaZ**
Mean ± SD	13 ± 7	211 ± 74	13 ± 6	233 ± 80	12 ± 7	552 ± 193
N	695	281	619	251	1259	505
Minimum	3	69	3	83	2	35
Maximum	46	402	37	482	45	1323
WP2PuvrA	**Solvent Controls**	**5.0µg BP**	**Solvent Controls**	**5.0µg BP**	**Solvent Controls**	**1.0µg ENNG**
Mean ± SD	180 ± 31	944 ± 333	190 ± 32	856 ± 172	158 ± 35	1130 ± 544
N	654	158	610	142	1225	493
Minimum	101	75	107	332	50	249
Maximum	287	3041	297	1261	282	2924
TA 98	**Solvent Controls**	**1.0µg 2AA**	**Solvent Controls**	**1.0µg 2AA**	**Solvent Controls**	**1.0µg DR**
Mean ± SD	29 ± 9	1553 ± 689	31 ± 10	2505 ± 1125	23 ± 8	960 ± 625
N	667	275	613	247	1225	493
Minimum	8	153	5	568	4	50
Maximum	65	4019	71	4965	61	2716
TA 1537	**Solvent Controls**	**2.0µg 2AA**	**Solvent Controls**	**2.0µg 2AA**	**Solvent Controls**	**2.0µg ICR**
Mean ± SD	12 ± 8	233 ± 155	14 ± 8	386 ± 381	11 ± 7	121 ± 117
N	675	275	640	256	1249	505
Minimum	1	26	1	33	1	13
Maximum	34	692	39	4517	43	631

Sublimed Undensified Molybdenum Trioxide: In-vitro Micronucleus Assay in Human Lymphocytes

Study No. CTL/SV1230 conducted in 2004

by

Central Toxicology Laboratory
Alderley Park, Macclesfield,
Cheshire, United Kingdom

Prepared for:

International Molybdenum Association (**IMOA**)
4 Heathfield Terrace, Chiswick
London, W4 4JE, UK

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CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK

CTL/SV1230/REGULATORY/REPORT

SUBLIMED UNDENSIFIED MOLYBDENUM TRIOXIDE: *IN VITRO* MICRONUCLEUS ASSAY IN HUMAN LYMPHOCYTES

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SUBLIMED UNDENSIFIED MOLYBDENUM TRIOXIDE: *IN VITRO* MICRONUCLEUS ASSAY IN HUMAN LYMPHOCYTES

STUDY DETAILS

Sponsor:	International Molybdenum Association 2 Baron's Gate 33 Rothschild Road London W4 5HT
Sponsor Reference:	CO3162/3
CTL Test Substance Reference Number:	Y12618/001
CTL Study Number:	SV1230
Document Number:	CTL/SV1230/REG/REPT

AUTHOR

V Fox

DATE OF ISSUE

6 May 2005

STATEMENT OF DATA CONFIDENTIALITY CLAIM

THIS DOCUMENT CONTAINS INFORMATION CONFIDENTIAL AND TRADE SECRET TO INTERNATIONAL MOLYBDENUM ASSOCIATION.

It should not be disclosed in any form to an outside party, nor should information contained herein be used by a registration authority to support registration of this product or any other product without the written permission of International Molybdenum Association.

STATEMENT OF GLP COMPLIANCE AND AUTHENTICATION

I, the undersigned, declare that the objectives laid down in the protocol were achieved and that the data generated are valid. The report fully and accurately reflects the procedures used and the raw data generated in the above study.

The study (SV1230) was conducted in compliance with the UK Principles of Good Laboratory Practice (The United Kingdom GLP Regulations 1999, Statutory Instrument No. 3106, as amended 2004, Statutory Instrument No.994) except for the deviation listed below. These Principles are in accordance with the OECD Principles of Good Laboratory Practice, revised 1997 (ENV/MC/CHEM(98)17).

The following GLP deviation is considered not to affect the integrity of the study or the validity of the conclusions drawn:

(i) the stability, homogeneity and achieved concentration of the test substance in the vehicle used were not determined by analysis.

V Fox ..... 6 May 2005
Study Director Date

This page may be
Required by some
Regulatory Authorities

QUALITY ASSURANCE STATEMENT

In accordance with CTL policy and QA procedures for Good Laboratory Practice, this report has been audited and the conduct of this study has been inspected as follows:

Date	Audit/Inspection	Date of QA Report
05 Mar 2004	Slide preparation	09 Mar 2004
24 May 2004	Draft report	28 May 2004
04 May 2005	Final report review	05 May 2005

In addition, inspections associated with this type of study were made as follows:

15 Jan 2004	Protocol	15 Jan 2004
21 Jan 2004	Slide coding	22 Jan 2004
22 Jan 2004	Slide scoring	22 Jan 2004
30 Jan 2004	Transfer	30 Jan 2004
12 Feb 2004	Dilutions	13 Feb 2004
04 Mar 2004	Dose administration	04 Mar 2004
18 Mar 2004	Dose preparation	18 Mar 2004
18 Mar 2004	Addition of S9	18 Mar 2004

Facilities and process based procedures associated with this type of study were inspected in accordance with QA Standard Operating Procedures.

So far as can be reasonably established, the methods described and the results given in the final report accurately reflect the raw data produced during the study, SV1230.

V L Wright ..... 6 May 2005

(CTL Quality Assurance Unit)

STUDY CONTRIBUTORS

The following contributed to this report in the capacities indicated:

Name	Title
V Fox	Study Director, CTL
B M Elliott	Study Reviewer

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1. SUMMARY

1.1 Study design

Sublimed Undensified Molybdenum Trioxide (CAS No. 1313-27-5) was evaluated for its clastogenic or aneugenic potential in an *in vitro* micronucleus assay in three separate experiments using human lymphocytes treated in the presence and absence of a rat liver-derived metabolic activation system (S9-mix). In Experiments 1 and 2, cultures were treated for a period of 3 hours both in the presence and absence of S9-mix. In an additional experiment (Experiment 3), cultures were treated for 3 hours in the presence and absence of S9-mix and 20 hours in the absence of S9-mix.

Cultures treated with Sublimed Undensified Molybdenum Trioxide at the following concentrations were selected for micronucleus analysis along with the appropriate solvent and positive control cultures.

Experiment 1		**Experiment 2**		**Experiment 3**		
+S9-mix 3 hour treatment	-S9-mix 3 hour treatment	+S9-mix 3 hour treatment	-S9-mix 3 hour treatment	+S9-mix 3 hour treatment	-S9-mix 3 hour treatment	-S9-mix 20 hour treatment
1439µg/ml	1439µg/ml	1000µg/ml	1000µg/ml	1439µg/ml	1439µg/ml	1000µg/ml
1000µg/ml	1000µg/ml	500µg/ml	500µg/ml	1000µg/ml	1000µg/ml	500µg/ml
250µg/ml	250µg/ml	100µg/ml	100µg/ml	250µg/ml	250µg/ml	100µg/ml

1.2 Results

The highest concentration selected for micronucleus analysis was 10mM (1439µg/ml), which is the limit concentration for the assay, or was limited by toxicity.

Reductions in mitotic activity were observed in cultures from all experiments, thus demonstrating that Sublimed Undensified Molybdenum Trioxide is biologically active in this test system.

Precipitation of the test substance in the culture medium was visible on the slides of cultures treated with Sublimed Undensified Molybdenum Trioxide at concentrations of 1000µg/ml and above.

The culture medium was at an acceptable pH (within the normal control culture range) for the duration of treatment with the test substance. Changes in pH have been reported to result in the production of chromosomal aberrations (Scott *et al*, 1991). As the test substance was acidic, the pH of the stock test substance was adjusted to between 7.0 and 7.3 using sodium hydroxide and checked at the end of the treatment periods.

Statistically significant increases in the percentage of aberrant cells, compared to the solvent control, were recorded at 1439µg/ml in Experiment 1 both in the presence and absence of S9-mix.

No statistically significant increases in micronucleated binucleate cells, compared to the solvent control values, were recorded in Experiment 2 in either the presence or absence of S9-mix.

As the highest concentration used in Experiment 2 was not as high as that used in Experiment 1, a third experiment was conducted to clarify the results obtained in Experiment 1. In Experiment 3, cultures were treated for 3 hours in the presence and absence of S9-mix and 20 hours in the absence of S9-mix. No statistically significant increases in the incidence of micronucleated binucleate cells, compared to the solvent control values, were recorded in Experiment 3 showing that the increases in micronuclei observed in Experiment 1 were not reproducible.

The sensitivity of the test system, and the metabolic activity of the S9-mix employed, were clearly demonstrated by the increases in micronucleated binucleate cells induced by the positive control agents, mitomycin C and cyclophosphamide.

1.3 Conclusion

It is concluded that, under the conditions of this assay, Sublimed Undensified Molybdenum Trioxide is not reproducibly clastogenic or aneugenic to cultured human lymphocytes treated *in vitro* in either the presence or absence of S9-mix.

2. INTRODUCTION

2.1 Purpose

The purpose of this study was to investigate whether the test substance induced genetic damage (clastogenic as a result of chromosome breakage or aneugenic as a result of chromosome lagging) *in vitro* in the human peripheral blood lymphocyte micronucleus assay.

2.2 Regulatory guidelines

There are no specific Regulatory Guidelines relating to studies of this type. The study design is based on the recommendations of Kirsh-Volders *et al* (2003).

2.3 Justification for test system selection

The *in vitro* human lymphocyte micronucleus assay is designed to detect chemically induced changes in cells treated in culture. The human peripheral lymphocyte used is a sensitive target cell for the induction of *in vitro* genetic damage when stimulated to provide large numbers of rapidly dividing cells in culture. When the cells are in exponential growth they are exposed to various concentrations of the test substance. The cells are harvested, slides are prepared and analysed for the presence or absence of micronucleated binucleate cells. Since micronuclei can also be seen in solvent control cultures, the assay is based on the observation of an increased incidence of micronucleated cells over and above that seen in the solvent control cultures.

Molybdenum salts have been reported to induce micronuclei *in vitro* in human lymphocytes (Titenko-Holland *et al*; 1998) and syrian hamster embryo cells (Gibson *et al*; 1997). In neither of these studies was any adjustment made for possible changes in pH. Changes in pH have been reported to result in the production of chromosomal aberrations (Scott *et al*, 1991). As the test substance was acidic, the pH of the stock test substance was adjusted to between 7.0 and 7.3 using sodium hydroxide and checked at the end of the treatment periods.

2.4 Study dates

The study was initiated on 3 February 2004. The experimental phase started on 3 February 2004 and was completed on 8 April 2004.

2.5 Data storage

An original report, the study protocol and all raw data, samples and specimens pertaining to this study are retained in the CTL Archives, Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK for a minimum of 5 years from the date of issue of the final report. At the end of this period the Sponsor will be contacted regarding the further retention, return to the Sponsor or destruction of the archived materials.

3. TEST AND CONTROL SUBSTANCES

3.1 Test substance

Name:	Sublimed Undensified Molybdenum Trioxide (CAS No. 1313-27-5)
Source:	IMOA
Colour:	Pale green
Physical state:	Powder
Batch reference:	Lot 1MS
CTL test substance reference number:	Y12618/001
Purity:	99.9% w/w
Storage conditions:	Cool, dark well ventilated area
Expiry date:	In excess of 1 year

From information supplied by the Sponsor, the test substance was used within the expiry date.

The characterisation of the test substance is the responsibility of the Sponsor.

In all cases where the concentration of test substance is quoted, this concentration refers to the concentration of test substance not corrected for purity.

3.2 Control substances

The control substance and solvent for the test sample was supplemented RPMI-1640 culture medium (CTL test substance reference number Y08056/001).

The positive control substances, mitomycin C (CTL test substance reference number Y02201/004) and cyclophosphamide (CTL test substance reference number Y01259/042) were obtained from Sigma Chemical Company.

4. EXPERIMENTAL PROCEDURES

4.1 Dosing preparations

An individual stock suspension of the test substance was prepared for each experiment in supplemented RPMI-1640 culture medium and the pH was adjusted to 7.0 in Experiment 1 to neutralise the culture medium and 7.3 in Experiments 2 and 3 (which is closer to the normal pH of culture medium) using sodium hydroxide. Serial dilutions were carried out as required in each case.

Both positive control substances were prepared as solutions in sterile double deionised water (CTL test substance reference number Y04517/035).

All test and positive control substance dosing preparations were prepared as close to the time of culture treatment as possible and were dosed at 8ml/10ml culture following removal of 8ml of the culture medium.

4.2 Analysis of dosing preparations

In view of the short term nature of studies of this type, no analyses of stability, homogeneity or achieved concentration were carried out on the preparations of the test or positive control substances either prior to or after addition to the human peripheral blood lymphocyte cultures.

4.3 Experimental design

Duplicate human peripheral blood cultures were exposed to the solvent, test substance or positive control substances at appropriate concentrations in the following experiments:

a) An *in vitro* micronucleus experiment (Experiment 1) was conducted using a sample of pooled blood. Cells were exposed to the test substance and control substances for a

period of 3 hours, both in the presence and absence of S9-mix. Solvent, untreated and positive control cultures were included.

b) A second independent *in vitro* micronucleus experiment (Experiment 2) was conducted using a sample of pooled blood. Cells were exposed to the test substance and control substances for a period of 3 hours, both in the presence and absence of S9-mix. Solvent, untreated and positive control cultures were included.

c) A third independent *in vitro* micronucleus experiment (Experiment 3) was conducted using a sample of pooled blood. Cells were exposed to the test substance and control substances for a period of 3 hours in the presence and absence of S9-mix and 20 hours in the absence of S9-mix. Solvent, untreated and positive control cultures were included.

Treatment of the cultures started approximately 24 hours after culture initiation. A single sampling time, 68 hours after culture initiation, was used.

In all three experiments a range of concentrations of Sublimed Undensified Molybdenum Trioxide was used in order to define suitable concentrations for micronucleus analysis.

4.4 Culture establishment

Human blood samples were obtained by venepuncture in lithium heparin tubes on the day prior to culture initiation or the day of culture initiation from healthy, non-smoking donors. Equal volumes of blood from 2 x 2 female donors were pooled together for Experiments 1 and 2 and blood from 2 male donors was pooled together for Experiment 3. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes and therefore an assumed low incidence of micronuclei caused by chromosomal breakage.

At 0 hours, cultures (10ml) were established by the addition of 0.5ml of whole blood to RPMI-1640 (Dutch modification) tissue culture medium supplemented with approximately 10% foetal bovine serum (FBS), 1.0IU/ml heparin, L-glutamine (2mM), 100IU/ml penicillin and 100µg/ml streptomycin. The lymphocytes were stimulated to enter cell division by addition of phytohaemagglutinin (PHA; at 5% v/v) and the cultures were maintained at approximately 37°C for 24 hours with gentle daily mixing where possible.

4.5 Metabolic activation system

The metabolic activation system (S9-mix) used in this study was prepared as required (on each day of culture treatment) as a 1:1 mixture of S9 fraction and cofactor solution.

S9 was purchased from MolTox Inc., and was prepared from male Sprague Dawley rats, dosed once daily (by oral gavage) for 3 days with a combined phenobarbital (80mg/kg bodyweight) and β-naphthoflavone (100mg/kg bodyweight) corn oil preparation. The treated animals were sacrificed on the day following the third dose. A 25% w/v homogenate (the S9 fraction) was prepared according to the method given in Callander *et al* (1995).

The cofactor solution was prepared as a single stock solution of Na_2HPO_4, KCl, glucose-6-phosphate, NADP (Na salt) and $MgCl_2$ (150 : 49.5 : 7.5 : 6 : 12mM) in sterile double deionised water and adjusted to a final pH of 7.4.

4.6 Culture treatment

Approximately 24 hours after culture establishment, the cultures were centrifuged and 8ml of the culture medium was removed and replaced with 8ml aliquots of supplemented culture medium containing the appropriate concentrations of the test substance. The culture medium was removed from the solvent control and positive controls and replaced with fresh supplemented medium. The positive controls were then treated with the appropriate concentration of cyclophosphamide or mitomycin C. In addition, 200µl of a 1:1 mix of S9 and co-factor solution was added to each culture to be treated in the presence of S9-mix.

Cultures from all experiments in the presence and absence of S9-mix were treated for a period of 3 hours at 37°C, after which the culture medium was removed following centrifugation and replaced with fresh supplemented RPMI-1640 culture medium. In addition, cultures from Experiment 3 in the absence of S9-mix were treated for a period of 20 hours, after which the culture was removed following centrifugation and replaced with fresh supplemented RPMI-1640 culture medium. The cultures were re-incubated at 37°C for the remainder of the 68 hour growth period.

The pH of the stock suspension of the test substance was adjusted to pH 7.0 in Experiment 1 and pH 7.3 in Experiments 2 and 3. In Experiment 1, the culture medium was neutralised to pH 7.0 and in subsequent experiments, it was decided to adjust the pH to a value representing

the normal pH of culture medium. The pH of the culture medium was checked in all experiments at the end of the treatment periods, using single treated cultures (minus blood, PHA and S9-mix) treated with the test substance. The osmolality of the culture medium was determined immediately following treatment in Experiment 2. The solubility of the test substance in the treated blood cultures and in media-only cultures was assessed by eye immediately after treatment and at the end of the treatment periods.

4.7 Culture harvesting

Approximately 24 hours prior to harvesting, the cultures were treated with cytochalasin B at a final concentration of 5.4µg/ml. Sixty-eight hours after culture establishment, the cultures were centrifuged, the supernatant was removed and the cells were treated with 5ml of pre-warmed (37°C) 0.075M KCl for approximately 5 minutes at room temperature. The blood culture/KCl was mixed whilst adding pre-cooled (4°C) freshly prepared methanol/glacial acetic acid fixative (3:1 v/v) dropwise and made up to a volume of approximately 10ml with methanol/acetic acid. The blood culture/KCl/fixative mixture was incubated for 10 minutes at room temperature. The fixative was removed following centrifugation and replaced with freshly prepared fixative. After at least two subsequent changes of fixative, slides were made by dropping the cell suspension on to clean, moist, labelled microscope slides. The slides were air dried, stained in filtered Giemsa stain (10% Gurr's R66 in buffered [pH 6.8] double deionised water) for 7 minutes, rinsed in water, air-dried and mounted with coverslips in DPX.

4.8 Slide analysis

Slides were examined to determine that they were of suitable quality and, where appropriate, the cytokinesis-block proliferation index (CBPI) was determined by examining 500 cells per culture. The CBPI is defined as:

1 x number of mononucleate cells
+ 2 x number of binucleate cells
+ 3 x number of tri- and tetranucleate cells
---
total number of cells scored

For each experiment, both in the presence and absence of S9-mix, duplicate cultures treated with Sublimed Undensified Molybdenum Trioxide at three concentrations were selected for micronucleus scoring along with the appropriate solvent and positive control cultures.

The slides were coded and five hundred binucleate cells were analysed from each selected culture for the incidence of micronuclei.

5. DATA EVALUATION

The Fisher Exact Probability Test (one-sided) was used to evaluate statistically the percentage of binucleate cells with micronuclei. Data from each treatment group, in the presence and absence of S9-mix, was compared with the respective solvent control group value. The data from each experiment have been interpreted as follows:

a) No statistically significant increase in micronucleated binucleate cells (at any concentration) above concurrent solvent control values - **NEGATIVE**.

b) A statistically significant increase in micronucleated binucleate cells, at least at one concentration - **POSITIVE**.

6. RESULTS

6.1 Determination of CBPI and selection of concentrations

Three independent *in vitro* micronucleus experiments were carried out using ranges of Sublimed Undensified Molybdenum Trioxide concentrations as detailed in Tables 1 - 2 which also detail the individual culture and mean CBPI and the number of binucleate cells observed for the solvent controls and the Sublimed Undensified Molybdenum Trioxide treated cultures.

The highest concentration selected for micronucleus analysis was 10mM (1439µg/ml), which is the limit concentration for the assay, or was limited by toxicity. Where the limit concentration was not selected, this was because the cultures at 1439µg/ml were considered

not to be suitable for micronucleus analysis due to lack of binucleate cells as a result of toxicity (Tables 1 - 2).

Reductions in mean binucleate index, compared to the solvent control values, were observed in cultures from both Experiment 1 (46% +S9-mix; 18% 3 hour -S9-mix), Experiment 2 (53% +S9-mix; 32%; 3 hour -S9-mix) and Experiment 3 (68% +S9-mix; 56% 3 hour -S9-mix, 73% 20 hour -S9-mix) treated with the highest concentrations of Sublimed Undensified Molybdenum Trioxide selected for micronucleus analysis.

Precipitation of the test substance in the culture medium was visible on the slides of cultures treated with Sublimed Undensified Molybdenum Trioxide at concentrations of 1000µg/ml and above.

The culture medium was at an acceptable pH (within the normal control culture range) for the duration of treatment with the test substance. Changes in pH have been reported to result in the production of chromosomal aberrations (Scott *et al*, 1991). As the test substance was acidic, the pH of the stock test substance was adjusted to between 7.0 and 7.3 using sodium hydroxide and checked at the end of the treatment periods (Table 5).

Treatment of the culture medium with Sublimed Undensified Molybdenum Trioxide up to 1439µg/ml (10mM) had no significant effect on osmolality (Table 6).

6.2 Results of micronucleus analyses

From the results detailed in Section 6.1, cultures treated with following concentrations of Sublimed Undensified Molybdenum Trioxide were selected for micronucleus analysis.

Experiment 1		**Experiment 2**		**Experiment 3**		
+S9-mix 3 hour treatment	-S9-mix 3 hour treatment	+S9-mix 3 hour treatment	-S9-mix 3 hour treatment	+S9-mix 3 hour treatment	-S9-mix 3 hour treatment	-S9-mix 20 hour treatment
1439µg/ml	1439µg/ml	1000µg/ml	1000µg/ml	1439µg/ml	1439µg/ml	1000µg/ml
1000µg/ml	1000µg/ml	500µg/ml	500µg/ml	1000µg/ml	1000µg/ml	500µg/ml
250µg/ml	250µg/ml	100µg/ml	100µg/ml	250µg/ml	250µg/ml	100µg/ml

The results of the micronucleus analysis are shown in Tables 3 – 4 expressed as the mean percentage of micronucleated binucleate cells at each concentration. Individual data from each culture are given in Tables 7 - 8.

Statistically significant increases in the percentage of aberrant cells, compared to the solvent control, were recorded at 1439µg/ml in Experiment 1 both in the presence and absence of S9-mix.

No statistically significant increases in micronucleated binucleate cells, compared to the solvent control values, were recorded in Experiment 2 in either the presence or absence of S9-mix.

Although the highest concentration used in Experiment 2 (1000µg/ml) was not as high as that used in Experiment 1 (1439µg/ml), the degree of biological activity (toxicity) as measured by reduction in the percentage of binucleate cells and CBPI, was comparable or greater. However, a third experiment was conducted to clarify the results. In Experiment 3, cultures were treated for 3 hours in the presence and absence of S9-mix and 20 hours in the absence of S9-mix. No statistically significant increases in the incidence of micronucleated binucleate cells, compared to the solvent control values, were recorded in Experiment 3.

The positive control materials, mitomycin C and cyclophosphamide induced statistically and biologically significant increases in the percentage of aberrant cells, compared to the solvent control cultures.

7. DISCUSSION

The test substance, Sublimed Undensified Molybdenum Trioxide, was assessed in an *in vitro* micronucleus assay in cultured human peripheral blood lymphocytes to determine if it induces genetic damage.

Sublimed Undensified Molybdenum Trioxide was tested over a range of concentrations, both in the presence and absence of S9-mix, in three independent cytogenetic tests.

Increases in micronucleated binucleate cells observed in Experiment 1 were not reproducible in Experiment 2 or in Experiment 3 in which the same concentration as that used in

Experiment 1 was evaluated for cultures treated for 3 hours in the presence and absence of S9-mix and a 20 hour treatment period in the absence of S9-mix was also used.

The sensitivity of the test system, and the metabolic activity of the S9-mix employed, were clearly demonstrated by the increases in the frequencies of aberrant cells induced by the positive control agents, mitomycin C and cyclophosphamide.

The data obtained in this study therefore show that the test sample of Sublimed Undensified Molybdenum Trioxide did not induce reproducible genetic damage in human peripheral blood lymphocytes following *in vitro* treatment in either the presence or absence of S9-mix.

8. CONCLUSION

It is concluded that, under the conditions of this assay, Sublimed Undensified Molybdenum Trioxide (CAS No. 1313-27-5) is not reproducibly clastogenic or aneugenic to cultured human lymphocytes treated *in vitro* in either the presence or absence of S9-mix.

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TABLE 1 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 1 – 3 hour treatment (-S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.664 1.510	1.587	260 215	238
Sublimed Undensified Molybdenum Trioxide (µg/ml)				
1439	1.422 1.406	1.414	199b 189b	194
1000	1.506 1.486	1.496	221b 221b	221
500	a a		a a	
250	1.682 1.674	1.678	265 271	268
175	a a		a a	
100	a a		a a	
50	a a		a a	
10	a a		a a	

a - CBPI and binucleate cell count not required for selection of concentrations for analysis
b - Precipitate of test substance visible on the slides

TABLE 1 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 2 – 3 hour treatment (-S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.654 1.734	1.694	301 331	316
Sublimed Undensified Molybdenum Trioxide (µg/ml)				
1439	1.160 b	1.160Δ	80c c	80
1000	1.474 1.392	1.433	231c 196c	214
500	1.682 1.648	1.665	311d 300d	306
250	a a			
175	a a		a a	
100	1.776 1.736	1.756	342 338	340
50	a a		a a	
10	a a		a a	

a - CBPI and binucleate cell count not required for selection of concentrations for analysis
b - Too few binucleate cells and excessive reduction in binucleate cells observed in Culture A
c - Precipitate of test substance visible on the slides
d - Light precipitate visible on the slides
Δ - Mean determined from a single culture

TABLE 1 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 3 – 3 hour treatment (-S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.514 1.524	1.519	245 244	245
Sublimed Undensified Molybdenum Trioxide (µg/ml)				
1439	1.206 1.230	1.218	103b 113b	108
1000	1.296 1.362	1.329	148b 179b	164
500	a a		a a	
250	1.510 1.584	1.547	229 264	247
175	a a		a a	
100	a a		a a	
50	a a		a a	
10	a a		a a	

a - CBPI and binucleate cell count not required for selection of concentrations for analysis
b - Precipitate of test substance visible on the slides

TABLE 1 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 3– 20 hour treatment (-S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.460 1.602	1.531	230 257	244
Sublimed Undensified Molybdenum Trioxide (µg/ml)				
1439	b b		bc bc	
1000	1.114 1.154	1.134	57c 77c	67
500	1.248 1.306	1.277	124 151	138
250	a a		a a	
175	a a		a a	
100	1.536 1.604	1.570	226 262	244
50	a a		a a	
10	a a		a a	

a - CBPI and binucleate cell count not required for selection of concentrations for analysis
b - Too few binucleate cells for micronucleus analysis
c - Precipitate of test substance visible on the slides

TABLE 2 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 1 – 3 hour treatment (+S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.534 1.444	1.489	229 200	215
Sublimed Undensified Molybdenum Trioxide (µg/ml)				
1439	1.222 1.242	1.232	111b 121b	116
1000	1.460 1.478	1.469	208b 226b	217
500	a a		a a	
250	1.526 1.508	1.517	237 228	233
175	a a		a a	
100	a a		a a	
50	a a		a a	
10	a a		a a	

a - CBPI and binucleate cell count not required for selection of concentrations for analysis
b - Precipitate of test substance visible on the slides

TABLE 2 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 2 – 3 hour treatment (+S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.784 1.790	1.787	338 327	333
Sublimed Undensified Molybdenum Trioxide (µg/ml)				
1439	b b		bc bc	
1000	1.320 1.308	1.314	160c 154c	157
500	1.524 1.544	1.534	242d 246d	244
250	a a		a a	
175	a a		a a	
100	1.714 1.744	1.729	301 316	309
50	a a		a a	
10	a a		a a	

a - CBPI and binucleate cell count not required for selection of concentrations for analysis
b - Too few binucleate cells for micronucleus analysis
c - Precipitate of test substance visible on the slides
d - Light precipitate visible on the slides

TABLE 2 - CYTOKINESIS-BLOCK PROLIFERATIVE INDICES IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 3 – 3 hour treatment (+S9-mix)				
Treatment	Cytokinesis-Block Proliferative Index %	Mean Cytokinesis-Block % Proliferative Index	No. of binucleate cells/500 cells	Mean binucleate cells/500 cells
Solvent Control (10µl/ml)	1.696 1.690	1.693	250 275	263
Sublimed Undensified Molybdenum Trioxide (µg/ml)				
1439	1.158 1.182	1.170	79b 91b	85
1000	1.368 1.386	1.377	174b 187b	181
500	a a		a a	
250	1.494 1.528	1.511	223 230	227
175	a a		a a	
100	a a		a a	
50	a a		a a	
10	a a		a a	

a - CBPI and binucleate cell count not required for selection of concentrations for analysis
b - Precipitate of test substance visible on the slides

TABLE 3 - MEAN MICRONUCLEATED BINUCLEATE CELLS AND REDUCTION IN BINUCLEATE CELLS IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

Treatment		Mean % Micronucleated binucleate Cells	Mean % Reduction in binucleate cells
Experiment 1 – 3 hour treatment			
Solvent Control		1.9	-
Mitomycin C	0.5µg/ml	20.0**	-
Sublimed Undensified Molybdenum Trioxide	1439µg/ml	4.2**	18
	1000µg/ml	1.5	7
	250µg/ml	1.7	No reduction
Experiment 2 – 3 hour treatment			
Solvent Control		0.4	-
Mitomycin C	0.5µg/ml	22.0**	-
Sublimed Undensified Molybdenum Trioxide	1000µg/ml	0.4	32
	500µg/ml	1.0	3
	100µg/ml	0.8	No reduction
Experiment 3 – 3 hour treatment			
Solvent Control		0.7	-
Mitomycin C	0.5µg/ml	23.8**	-
Sublimed Undensified Molybdenum Trioxide	1439µg/ml	1.0	56
	1000µg/ml	0.4	33
	250µg/ml	0.6	No reduction
Experiment 3 – 20 hour treatment			
Solvent Control		1.0	-
Mitomycin C	0.25µg/ml	28.0**	-
Sublimed Undensified Molybdenum Trioxide	1000µg/ml	0.8	73
	500µg/ml	0.6	43
	100µg/ml	0.6	No reduction

** Statistically significant increase in the percentage of aberrant cells at $p<0.01$ using Fisher's Exact Test (one-sided).

Δ Positive control mitotic index and % aberrant cells are determined from a single culture.

TABLE 4 - MEAN MICRONUCLEATED BINUCLEATE CELLS AND REDUCTION IN BINUCLEATE CELLS IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

Treatment		Mean % Micronucleated binucleate Cells	Mean % Reduction in binucleate cells
Experiment 1 – 3 hour treatment			
Solvent Control		0.7	-
Cyclophosphamide	25µg/ml	6.0**	-
Sublimed Undensified Molybdenum Trioxide	1439µg/ml	3.7**	46
	1000µg/ml	1.4	No reduction
	250µg/ml	1.5	No reduction
Experiment 2 - 3 hour treatment			
Solvent Control		0.9	-
Cyclophosphamide	25µg/ml	8.0**	-
Sublimed Undensified Molybdenum Trioxide	1000µg/ml	0.5	53
	500µg/ml	0.4	27
	100µg/ml	0.5	7
Experiment 3 – 3 hour treatment			
Solvent Control		0.7	-
Cyclophosphamide	50µg/ml	12.2**	-
Sublimed Undensified Molybdenum Trioxide	1439µg/ml	0.6	68
	1000µg/ml	0.4	31
	250µg/ml	0.6	14

** Statistically significant increase in the percentage of aberrant cells at $p<0.01$ using Fisher's Exact Test (one-sided).

Δ Positive control mitotic index and % aberrant cells are determined from a single culture.

TABLE 5 - pH DATA

Treatment	Experiment 1	Experiment 2		Experiment 3		
	After 3 hrs	After treatment	After 3 hrs	After treatment	After 3 hrs	After 20 hrs
Solvent Control	7.09	7.13	7.16	7.11	7.12	7.18
Sublimed Undensified Molybdenum Trioxide						
1439µg/ml	6.95	7.04	6.95	7.19	7.13	7.17
1000µg/ml	7.11	7.09	7.00	7.22	7.18	7.23
500µg/ml	7.22	7.17	7.14	7.20	7.17	7.23
250µg/ml	7.28	7.21	7.18	7.20	7.18	7.24
175µg/ml	7.32	7.23	7.22	7.20	7.21	7.26
100µg/ml	7.36	7.25	7.24	7.21	7.20	7.26
50µg/ml	7.35	7.25	7.23	7.20	7.19	7.25
10µg/ml	7.30	7.23	7.21	7.16	7.16	7.23

Stock formulation in Experiment 1 adjusted to pH 7.0

Stock formulation in Experiments 2 and 3 adjusted to pH 7.3 as this as this is closer to the normal pH of culture medium

Culture medium pH not adjusted either prior to treatment of the control cultures or prior to use in the serial dilutions of the stock test substance formation

TABLE 6 - OSMOLALITY DATA

Treatment	Osmolality (mmol/kg) Recorded in Experiment 2
Solvent Control	302
Sublimed Undensified Molybdenum Trioxide	
1439µg/ml	304
1000µg/ml	302
500µg/ml	301
250µg/ml	299
175µg/ml	305
100µg/ml	302
50µg/ml	305
10µg/ml	303

TABLE 7 - INDIVIDUAL CULTURE DATA IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 1 - 3 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	9
		B	500	10
Mitomycin C	0.5µg/ml	A	500	100
Sublimed Undensified Molybdenum Trioxide	1439µg/ml	A	500	23
		B	500	19
	1000µg/ml	A	500	10
		B	500	5
	250µg/ml	A	500	9
		B	500	8

EXPERIMENT 2 – 3 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	2
		B	500	2
Mitomycin C	0.5µg/ml	A	500	110
Sublimed Undensified Molybdenum Trioxide	1000µg/ml	A	500	3
		B	500	1
	500µg/ml	A	500	9
		B	500	1
	100µg/ml	A	500	4
		B	500	4

TABLE 7 - INDIVIDUAL CULTURE DATA IN THE ABSENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 3 - 3 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	3
		B	500	4
Mitomycin C	0.5µg/ml	A	500	119
Sublimed Undensified Molybdenum Trioxide	1439µg/ml	A	500	5
		B	500	5
	1000µg/ml	A	500	2
		B	500	2
	250µg/ml	A	500	2
		B	500	4

EXPERIMENT 3- 20 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	3
		B	500	7
Mitomycin C	0.25µg/ml	B	500	140
Sublimed Undensified Molybdenum Trioxide	1000µg/ml	A	500	3
		B	500	5
	500µg/ml	A	500	4
		B	500	2
	100µg/ml	A	500	5
		B	500	1

TABLE 8 - INDIVIDUAL CULTURE DATA IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 1 - 3 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	4
		B	500	3
Cyclophosphamide	25µg/ml	A	500	30
Sublimed Undensified Molybdenum Trioxide	1439µg/ml	A	500	23
		B	500	14
	1000µg/ml	A	500	7
		B	500	7
	250µg/ml	A	500	10
		B	500	5

EXPERIMENT 2 - 3 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	5
		B	500	4
Cyclophosphamide	25µg/ml	B	500	40
Sublimed Undensified Molybdenum Trioxide	1000µg/ml	A	500	2
		B	500	3
	500µg/ml	A	500	1
		B	500	3
	100µg/ml	A	500	2
		B	500	3

TABLE 8 - INDIVIDUAL CULTURE DATA IN THE PRESENCE OF METABOLIC ACTIVATION (S9-MIX)

EXPERIMENT 3 - 3 hour treatment

Treatment	Concentration	Culture	No of Cells Examined	Number of Binucleate Cells with Micronuclei
Solvent Control	10µl/ml	A	500	3
		B	500	4
Cyclophosphamide	50µg/ml	A	500	61
Sublimed Undensified Molybdenum Trioxide	1439µg/ml	A	500	3
		B	500	3
	1000µg/ml	A	500	2
		B	500	2
	250µg/ml	A	500	2
		B	500	4

Mutation Research, 257 (1991) 147–204

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ADONIS 016511109100058R

MUTREV 07293

INTERNATIONAL COMMISSION FOR PROTECTION AGAINST ENVIRONMENTAL MUTAGENS AND CARCINOGENS

Genotoxicity under extreme culture conditions

A Report from ICPEMC Task Group 9

David Scott [a] (Chairman), Sheila M. Galloway [b], Richard R. Marshall [c], Motoi Ishidate Jr. [d], David Brusick [e], John Ashby [f] and Brian C. Myhr [g]

[a] *Cancer Research Campaign Laboratories, Paterson Institute for Cancer Research, Manchester (Great Britain),* [b] *Merck, Sharp and Dohme Research Laboratories, West Point, PA (U.S.A.),* [c] *Hazleton Microtest, York (Great Britain),* [d] *National Institute of Hygienic Sciences, Tokyo (Japan),* [e] *Hazleton Laboratories America, Kensington, MD (U.S.A.),* [f] *Imperial Chemical Industries, Manchester (Great Britain) and* [g] *Hazleton Laboratories America, Kensington, MD (U.S.A.)*

(Received 2 August 1990)
(Accepted 2 August 1990)

Keywords: Culture conditions, extreme; Genotoxicity, extreme culture conditions

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Correspondence: Dr. J.D. Jansen, Scientific Secretary ICPEMC, c/o TNO Medical Biological Laboratory, P.O. Box 45, 2280 AA Rijswijk (The Netherlands).

ICPEMC is affiliated with the International Association of Environmental Mutagen Societies (IAEMS) and the Institut de la Vie.

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1. Introduction

Chemicals that are genotoxic in vitro in mammalian cells often fail to give positive results when tested in vivo in experimental animals. For example, approximately 50% of chemicals that are clastogenic in vitro do not induce chromosome aberrations or micronuclei in the bone marrow of rodents (Thompson, 1986; Ishidate et al., 1988). There are many possible reasons for such discrepancies (see Waters et al., 1988) including: (a) the absence of detoxicification or excretion processes in vitro; (b) metabolic processes, unique to the cells used in in vitro bioassays, that create active genotoxins; (c) the insensitivity of in vivo assays; (d) the use of conditions for in vitro tests that are so extreme and artificial as to be irrelevant to the situation in vivo.

This Report addresses the latter possibility. Four in vitro conditions have been considered as possibly generating such 'false positive' results: excessively high concentrations, high levels of cytotoxicity, the use of metabolic activation systems which in themselves may be genotoxic, and extremes of pH. Most of the data available relate to clastogenesis but other genotoxic endpoints have been considered when appropriate.

2. Genotoxicity at high concentrations in vitro

Genotoxicity assays in vitro are often conducted at concentrations up to the maximum solubility of the test compound. Indeed such a procedure is recommended in several Guidelines (Table 1, for clastogenesis) to optimise detection. For highly soluble, relatively non-toxic substances this can mean testing chemicals at tens of milligrams per millilitre or up to almost molar concentrations in the test medium. Concern has been expressed that at such high concentrations of test agent the assay system will itself become subverted, i.e., disturbances to chromatin structure may result from the test chemical perturbing cellular homeostasis, rather than itself directly modifying chromatin structure. Further, such effects may have no relevance to the situation in vivo, particularly to human exposure, because of the high cellular concentrations required.

2.1. Genotoxicity associated with high osmolality

Ishidate et al. (1984) were the first to suggest that the clastogenicity of certain chemicals (e.g. sucrose, propylene glycol) at high doses might be a consequence of the elevated osmolality of the culture medium rather than to the test compounds themselves. More recently, several chemicals, which are probably not DNA-reactive, have been found to induce a variety of genotoxic effects (e.g. clastogenesis, mutations at the TK locus in mouse lymphoma cells, DNA-strand breakage and morphological transformation) at high osmolality (Table 2). None of these agents induce gene mutations in Ames tests, but sodium and potassium chloride induce base substitutions and frameshift mutations in yeast at the very high concentration of 2000 mM (Parker and von Borstel, 1987).

Few of these studies have been sufficiently extensive to establish accurate dose/response relationships but a threshold response is suggested by some investigations (e.g. Ishidate et al., 1984; Ashby and Ishidate, 1986). The lowest concentration at which putative osmolality-related genotoxicity has so far been observed is 19.5 mM (4.0 mg/ml) of sodium saccharin which induces chro-

TABLE 1

GUIDELINES ON EXPOSURE CONCENTRATIONS IN CLASTOGENICITY TESTS IN VITRO

Recommendation	Source
'...the highest dose suppressing the mitotic activity by approximately 50%'	Health and Safety Commission (1982)
'The highest test substance concentration...should suppress mitotic activity by approximately 50 percent. Relatively insoluble substances should be tested *up to the limit of solubility*. For freely-soluble non-toxic substances the *upper test substance concentration should be determined on a case-by-case basis*.'	Organisation for Economic Cooperation and Development (1983)
'The highest dose chosen for testing should be one which causes a significant reduction in mitotic index... Agents that are non-cytotoxic should be tested *up to their maximum solubility*.'	United Kingdom Environmental Mutagen Society (1983)
'...the highest dose suppressing the mitotic activity by approximately 50%.'	European Communities (1984)
'Generally the highest test substance concentration...should show evidence of cytotoxicity or reduced mitotic activity. Relatively insoluble substances should be tested *up to the limit of solubility*. For freely soluble nontoxic chemicals, *the upper test chemical concentration should be determined on a case by case basis*'	USA Environmental Protection Agency (1985, 1987)
'Perform the test with the concentration of the test substance at which it produced a 50% or greater inhibition of cell growth or mitosis at the maximum dose level... In case of a test substance devoid of cytotoxic activity, *a concentration of 5 mg/ml (or equivalent of 10 mM)* should be employed at the maximum dose level...'	Japanese Guidelines (1987)
'For agents where no cytotoxicity can be demonstrated a...maximum of... *5 mg/ml* is frequently used. Use as a maximum concentration one that reduces MI and PI (proliferation index) by about 50%'	American Society for Testing and Materials (see Preston et al., 1987)

mosome aberrations in CHL cells (Ashby and Ishidate, 1986). Typically such genotoxic effects are observed when the osmolality of the culture medium increases by > 100 milliosmoles/kg. However, there is no simple relationship between osmolality and clastogenesis when all chemicals are considered and Marzin et al. (1986) detected a significant increase in aberrations in human lymphocytes treated with urea with an increase in osmolality of less than 50 mOsm/kg (from 275 to 320 mOsm/kg).

Genotoxic effects that are found only at high levels of osmolality are unlikely to occur in humans. Although sodium saccharin (see above) is weakly mutagenic in rodents (Ashby, 1985) this is at doses of about 10 g/kg, and an effect is demonstrable only because the chemical is tolerated at high levels in experimental animals ($LD_{50} = 17$ g/kg in mice). Brusick (1987) speculated that results from some cancer studies in rodents where dietary levels of the materials would lead to high consumption of sodium or potassium ions could be interpreted solely on the basis of ion levels in the target organ (typically the urinary bladder or kidney). Further research will be required to determine if hyper-osmolality can induce chromosome aberrations in vivo which might lead to tumour induction in experimental animals or whether other alterations in target organs are responsible. However, the relevance of such observations to human exposure and consequent risk has been seriously questioned (Ashby, 1985).

Osmotic effects are induced by diffusable molecules, and these can be ionic (e.g. NaCl) or neutral (e.g. glycerol). It is, at this stage, not possible to separate the several possible mechanisms by which high-dose genotoxicity may be produced. For example, the effects produced by high dose-levels of sodium saccharin may represent the result of non-specific osmotic effects, or the intracellular presence of high concentrations of ionic species, or the presence of high concentrations of sodium ions — the latter explanation being able to also accommodate the clastogenicity of sodium chlo-

TABLE 2

GENOTOXIC EFFECTS IN VITRO ASSOCIATED WITH HIGH OSMOLALITY OF THE CULTURE MEDIUM

Chemical	Endpoint	Cell type	Result	LEC			Treatment time (h)	Toxicity at LEC	Comment	Reference
				mg/ml	mM	mOsm/kg				
Calcium saccharin	Chromosome aberrations	CHL	+	8.0	19.8	327	24, 48			Ashby and Ishidate (1986)
Dimethyl sulphoxide	Mutation (TK)	Mouse lymphoma	+	108	1390	2383	4	Total growth 49% of control		Wangenheim and Bolcsfoldi (1988)
Ethylene glycol	Chromosome aberrations	CHL	+	60	968		48			Ishidate (1988)
		CHL(+S9)	+	20	322		6			Ishidate (1988)
	Gene mutation	Salmonella	−							Ishidate (1988)
Glucose	Mutation (TK)	Mouse lymphoma	+	37	204	350	4	Total growth 43% of control		Wangenheim and Bolcsfoldi (1988)
Magnesium chloride	Chromosome aberrations	CHL	+	8.0	59.0		24, 48			Ashby and Ishidate (1986)
Magnesium saccharin	Chromosome aberrations	CHL	+	8.0	20.6		24, 48			Ashby and Ishidate (1986)
D-Mannitol	Chromosome aberrations	Human lymphocytes	+	13.6	75	370	24	M.I. = 70% of control		Marzin et al. (1986)
		CHL	−				24, 48		No significant increase up to 11 mM	Ishidate (1988)
	Gene mutation	Salmonella	−							Ishidate (1988)
Potassium chloride	Chromosome aberrations	CHO	+	6.0	80	445	22	CFE = 45%		Galloway et al., in Brusick (1986)
			+	10.4	140	527	4	CFE = 71%		Galloway et al. (1987a)
			+	5.6	75	425	24	CFE = 44% MI = 46% of control		Seeberg et al. (1988)
		CHO (+S9)	+	3.7	49	536	4	CFE = 28% MI = 112% of control		Seeberg et al. (1988)
		CHL	−	4.0	53	405	24, 48			Ishidate (1988)
	SCE	CHO	±	13.4	180	626	4		Cell density effect? (see footnote)	Galloway et al. (1987a)
	Mutation (TK)	Mouse lymphoma (±S9)	+	4.0	54			Total growth 26–69% of control		Myhr, in Brusick (1986)
	Mutation (HPRT)	V79	+	5.6	75	425	3	None	Effective over narrow dose range	Seeberg et al. (1988)
		V79 (+S9)	+	2.8	37.5	357	3		Poorly reproducible. Effective over narrow dose range	Seeberg et al. (1988)

	Gene mutation	Salmonella (5 strains ±S9)	−						No mutants at 1.9–30 mg per plate	Seeberg et al. (1988)
	Gene mutation	Saccharo-myces	+	150	2000		1.0	CFE = 67%	No mutation in stationary phase cells	Parker and Von Borstel (1987)
	Transformation	Balb/c-3T3	+	4.0	54	400				Rundell and Matthews, in Brusick (1986)
	UDS	HeLa (±S9)	−						No UDS up to 200 mM	Seeberg et al. (1988)
	DNA ssb	CHO	+	15	200	689	4	No direct measurement but 160 mM gave CFA = 3% in same series of experiments		Galloway et al. (1987a)
	DNA dsb	CHO	−				4		No dsb detected up to 260 mM (756 mOsm/kg)	Galloway et al. (1987a)
Potassium saccharin	Chromosome aberrations	CHL	+	8.0	36.0	347	24, 48			Ashby and Ishidate (1986)
Polyethylene glycol	Mutation (TK)	Mouse lymphoma	+	150	20	> 620	4	Total growth 84% of control		Wangenheim and Bolcsfoldi (1988)
Propylene glycol	Chromosome aberrations	CHL	+	32	421	534	48			Ishidate (1988)
		CHL (+S9)	−	64	842	1000	3			Ishidate (1988)
	Gene mutation	Salmonella	−							Ishidate (1988)
Sodium chloride	Chromosome aberrations	CHL	+	7.0	120	512	24			Ishidate et al. (1984)
		CHO	+	8.2	140	560	22	CFE = 40%		Galloway et al., in Brusick (1986)
			+	8.8	150	559	4	CFE = 89%		Galloway et al. (1987)
			+	8.8	150	559	24	CFE = 12% MI = 3% of control		Seeberg et al. (1988)
		CHO (+S9)	+	11.7	200	643	4	CFE = 36% MI = 97% of control		Seeberg et al. (1988)
		Human Lymphocytes	+	2.7	50	372	24	MI = 40% of control		Marzin et al. (1986)

TABLE 2 (continued)

Chemical	Endpoint	Cell type	Result	LEC mg/ml	LEC mM	LEC mOsm/kg	Treatment time (h)	Toxicity at LEC	Comment	Reference
	Micronuclei	Human fibroblasts	+	2.5	43		48	MI = 90% of control		Scott and Roberts (1987)
	SCE	CHO	−				4		25% increase above control at 250 mM (754 mOsm/kg) but not significant (see footnote)	Galloway et al. (1987a)
	Mutation (TK)	Mouse lymphoma	+	4.0	68			Total growth 20–40% of control		Myhr, in Brusick (1986)
		(±S9)	+	5.6	94.3	> 490	4	Total growth 25% of control		Wangenheim and Bolcsfoldi (1988)
	Mutation (HPRT)	V79 (±S9)	±				3		Sporadic increases at 100–150 mM (550–600 mOsm/kg)	Seeberg et al. (1988)
	Gene mutation	Salmonella (5 strains ±S9)	−						No mutants at 1.5–23 mg per plate	Seeberg et al. (1988)
	Gene Mutation	Saccharo-myces	+	117	2000		1.0	CFE = 65%	No mutations in stationary phase cells	Parker and von Borstel (1987)
	Transformation	Balb/c-3T3	+	3.3	57	400				Rundell and Matthews, in Brusick (1986)
	UDS	HeLa (±S9)	−				3		No UDS up to 200 mM	Seeberg et al. (1988)
	DNA ssb	CHO	+	21.9	375	937	4	No direct measurement but 200 mM gave CFE = 71% in same series of expts		Galloway et al. (1987a)
	DNA dsb	CHO	+	21.9	375	937	4	ditto		Galloway et al. (1987a)
Sodium saccharin	Chromosome aberrations	DON	+	10.3	50	322	26	'Considerable mitotic inhibition'		Abe and Sasaki (1977)
		CHL	+	4.0	19.5		24, 48			Ashby and Ishidate (1986)
	SCE	DON	±	1.0	4.8		26	'Considerable mitotic inhibition' see footnote	×2 spontaneous frequency. No dose response.	Abe and Sasaki (1977)
		CHO	±	1.0	4.8		24		4.8 mM induced <10% increase. 48 mM required to give increase up to 50%. See footnote	Wolff and Rodin (1978)

		Human lymphocytes	±	1.0	4.8		72		4.8 mM induced < 20% increase. 24 mM required to give increase > 50%. See footnote	Wolff and Rodin (1978)
		Human lymphocytes	±	10	48		24		70% increase with 48 mM See footnote	Brogger et al. (1979)
	Mutation (TK)	Mouse lymphoma (+S9)	±	17	83		4	Survival 7%	Positive only at highly toxic doses; significance doubtful	Clive et al. (1979)
	Gene mutation	Salmonella	−							Reviewed by Ashby (1985)
Sorbitol	Chromosome aberrations	CHO	+	55	300	610	4	CFE = 100%		Galloway et al. (1987a)
		CHL	−				24, 48		No significant increase up to 110 mM	Ishidate (1988)
	Gene mutation	Salmonella	−							Ishidate (1988)
Sucrose	Chromosome aberrations	CHL	+	70	204		24			Ishidate et al. (1984)
		CHO	+		200	529	22	CFE = 60%		Galloway et al. (1985)
			+	94	275	610	4	CFE = 60%		Galloway et al. (1987a)
	SCE	CHO	−				4		20% increase above control at 325 mM (671 mOsm/kg) but not significant	Galloway et al. (1987a)
	DNA ssb	CHO	−				4	No direct measurement but 325 mM gave CFE = 6% in same series of expts.	No ssb detected up to 350 mM (704 mOsm/kg)	Galloway et al. (1987a)
	DNA dsb	CHO	−				4		no dsb detected up to 400 mM (779 mOsm/kg)	Galloway et al. (1987a)
Urea	Chromosome aberrations	Human lymphocytes	+	3.0	50		72	14% pycnotic cells	Severe chromosome fragmentation	Oppenheim and Fishbein (1965)
			+	3.0	50	320	24	MI = 70% of control		Marzin et al. (1986)
		CHL	+	12.0	200	463	24			Ishidate (1988)
	Mutation (TK)	Mouse lymphoma	+	31.8	530	> 816		Total growth 24% of control		Wangenheim and Bolcsfoldi (1988)
	Gene mutation	Salmonella	−							Ishidate (1988)

Chemicals have been tested without S9 mix unless specified.
Galloway et al. (1987) suggest that the apparent induction of SCEs by hyperosmotic conditions may be a consequence of reduced cell density and increased BrdUrd uptake by remaining cells.
CFE, colony-forming efficiency. MI, mitotic index.

ride itself. Detailed studies will be required to define the mechanisms of action; all we have at present are rather diffuse empirical observations. Galloway et al. (1987a) have suggested that the intracellular disturbances produced by high dose treatments may lead to changes in chromatin structure and/or enzyme activity.

Not all chemicals are genotoxic at high osmolality (Ishidate et al., 1984; Galloway et al., 1987a). For example, glycerol is non-clastogenic in CHO cells even with an increase in osmolality of more than 1100 mOsm/kg, at a concentration of 1000 mM, probably because rapid equilibration across the cell membrane precludes excessive osmotic stress (Galloway et al., 1987a).

In an attempt to avoid the problems associated with high osmolality, the Japanese Guidelines (1987) for clastogenicity testing in vitro recommend the use of a maximum concentration level of 10 mM. The validity of this recommendation can be investigated by examining dose–response data in vitro for those chemicals which are clastogenic in vivo. The possibility of undertaking such an analysis has been facilitated by two recent literature reviews. Ishidate et al. (1988) have listed the lowest effective concentrations (LEC) for clastogenicity of 466 chemicals tested in a variety of mammalian cell types in vitro. (Designation of a lowest effective concentration does not necessarily imply the existence of a threshold. Extensive dose–response data are required for this purpose.) About 20% of these chemicals have also been tested for micronucleus induction in vivo in rodent bone marrow. Thompson published a similar paper in 1986 which was restricted to chemicals which had been tested both in vivo and in vitro and found to be positive in one or both tests. In Thompson's review the in vivo clastogenicity data included tests for both micronucleus *and* metaphase aberration induction in rodent marrow. However, LEC values for in vitro testing were not given. We have therefore listed from these reviews (Tables 3 and 4) those chemicals which have been tested both in vivo and in vitro, which are positive in one or both tests, where the in vivo endpoint is either micronucleus or metaphase aberration induction, and for which in vitro LEC data are available from the review of Ishidate et al. (1988).

2.2. Lowest effective concentrations for clastogenicity in vitro

Before considering those chemicals which have been tested both in vivo and in vitro (Tables 3 and 4) it is worthwhile examining the range of LEC values for all the in vitro clastogens reviewed by Ishidate et al. The LEC values of the 466 clastogens varied over more than 10 orders of magnitude, from 4.3×10^{-8} mM (Trenimon at 10^{-5} μg/ml) to 6.9×10^{2} mM (acetone at 4×10^{4} μg/ml); 125 (27%) had LEC values > 1.0 mM in all cell types tested and 37 (8%) had values > 10 mM. The latter group is listed in Table 6 and comprises a wide range of chemical species that are sufficiently soluble and non-toxic to enable cytogenetic data to be obtained at these high concentrations. Clastogenesis is not, however, an inevitable consequence of in vitro exposure to high concentrations of chemicals. Of 377 chemicals reported as non-clastogenic in the review of Ishidate et al., 91 were tested at > 10 mM.

A number of potentially DNA reactive agents are included in Table 6 because they have been tested without metabolic activation (e.g. diethanolnitrosamine), but others, even with activation (e.g. diethylnitrosamine, LEC 29 mM or 3 mg/ml), require these high doses for detection. Some chemicals (e.g. polyethylene glycol, urea) are probably clastogenic through osmotic effects. It should be noted that the majority of these chemicals have only been tested in Chinese hamster cells and have not been tested for activity in vivo (see right-hand column in Table 6).

2.2.1. LEC values in vitro for agents which are clastogenic in vivo

Table 3 lists 66 chemicals that induce metaphase aberrations and/or micronuclei in rodent bone marrow and have been tested for clastogenicity in vitro in a number of mammalian test systems whose descriptions and abbreviations are given in Table 5. All but 4 of the chemicals were clastogenic in vitro. Again, LEC values range over almost 10 orders of magnitude from 4.3×10^{-8} mM to 100 mM (Fig. 1). Thus, even for chemicals that are clastogenic in vivo, some require very high exposure concentrations to be detected in vitro in

some cell systems. In part, this probably reflects the inadequacies of metabolic activation systems.

From the data in Table 3 and Fig. 1 we have listed (Table 7) those chemicals whose clastogenicity would have been missed if upper concentration limits had been applied to in vitro tests. Upper limits of 1, 5, 10, 20 and 50 mM are considered. Reading vertically, one can see which chemicals would be missed if tested in particular cell types if a given upper limit was used for testing.

Of the 66 in vivo clastogens, 7 had LEC values in vitro of > 10 mM in at least one cell type or were non-clastogenic at > 10 mM. Since most testing laboratories use only one cell type for their in vitro clastogenicity assays, the potential of these 7 chemicals for in vivo clastogenesis might have been missed if an upper limit of 10 mM had been adopted for in vitro testing. However, the testing of these 7 chemicals may not in all cases have utilised suitable protocols; indeed many of the studies were not intended to establish LEC values. The data on these chemicals is evaluated below:

(a) Barbital. Tested only in Chinese hamster cells (CHL and DON) without metabolic activation. Positive in CHL at 11 mM after treatment for 48 h (negative at 24 h), inconclusive at the next lowest concentration (5.5 mM) for 48 h after an analysis of 100 cells (Ishidate and Odashima, 1977). Negative in DON after 26 h treatment at up to 8 mM (Abe and Sasaki, 1977).

Verdict: Probably detectable at < 10 mM in CHL if more cells were analysed. Possibly detectable at < 10 mM in DON cells with a longer treatment time. May require metabolic activation.

(b) Benzene. Detectable at < 10 mM in Chinese hamster cells only with metabolic activation [CHO (Palitti et al., 1985) and CHL (Ishidate and Sofuni, 1985)] and human lymphocytes with or without activation (Howard et al., 1985). Negative in RL4 (Priston and Dean, 1985) up to 12.8 mM (24 h). RL4 cells may not have the necessary metabolic capacity to activate benzene. Dean et al. (1985) caution that benzene floats on top of the medium so agitation of cultures is required and Proctor et al. (1986) have pointed out the high volatility of the chemical such that most is 'lost to the head space' in a closed treatment vessel.

Verdict: Probably detectable at < 10 mM in RL4 cells with activation.

(c) Dimethylaminobenzene. Positive in CHl-L at 0.11 mM (Lafi, 1985). Negative in CHO up to 55 mM even with activation, but the treatment time was only 1 h and sampling was at 12 h (Natarajan and Van Kesteren-van Leeuwen, 1981). Negative in RL4, but because of cytotoxicity it was possible only to test up to 0.36 mM (Malallah et al., 1982). Difficult to detect in bacterial mutation assays because of problems with metabolic activation (Parry and Arlett, 1985).

Verdict: May be detectable in CHO at < 10 mM with optimal conditions for metabolic activation and a later sampling time to allow for mitotic delay.

(d) Dimethylnitrosamine. Requires > 10 mM, with activation, for a positive result in CHO (Natarajan et al., 1976), human lymphocytes (HL) (Bimboes and Greim, 1976) and Syrian hamster fibroblasts (SHF) (Nishi et al., 1980). In CHL, LEC = 6.7 mM (Ishidate, 1988).

CHO: Doses used 8, 27 and 135 mM for 1 h. High yields of aberrations at 27 mM (152 per 100 cells); no significant increase at 8 mM but 6 exchanges per 100 cells observed.

Verdict: Probably detectable at 10 mM with a longer duration of treatment and analysis of 200 cells per sample.

HL: Only one dose used (50 mM) for 45 min. Low aberration yield (5 per 100 cells).

Verdict: May be detectable at < 10 mM with longer treatment time to ensure adequate activation.

SHF: Only one concentration used (100 mM for 3 h), which gave a high aberration yield (76 per 100 cells).

Verdict: May be detectable at < 10 mM.

Overall verdict: Probably detectable in various cell types, in addition to CHL, at < 10 mM with adequate protocols.

(e) Hexamethylphosphoramide (HMPA). Positive in HL (Ashby et al., 1985) and CH1-L (Danford, 1985) at < 10 mM. Positive in CHL at 33.5 mM without activation but inconclusive at 22.3 and 11.2 mM even after 200 cells analysed (Ishi-

TABLE 3

CHEMICALS WHICH INDUCE MICRONUCLEI OR METAPHASE ABERRATIONS IN RODENT BONE MARROW IN VIVO WHICH HAVE ALSO BEEN TESTED FOR CLASTOGENICITY IN VITRO

Data from Ishidate et al. (1988) and Thompson (1986).

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration (μg/ml)	Concentration (mM)	TRT (h)	REC (h)	Ref.
4	Acetaldehyde	75-07-0	[−M]	+	[6] Rat fibro	−	4.4	0.1	24	0	32
12 a	2-Acetylamino-	53-96-3	[+m+]	+	[4] CHL	+	500	2.2	3	21	174
b	fluorene			+	[4] CHL	−	2000	9	3	21	174
c				+	[6] RL1	−	30	0.13	24	0	61
d				−	[4] V79-4	−	5	0.022	24	0	227
25 a	Actinomycin D	50-76-0	[−m+]	+	[1] Hum lymph	−	1.8	0.0014	1	0	113
b				+	[1] Hum lymph	−	3.5	0.0028	1.5	0	47
c				+	[4] CHL	−	0.0125	0.00001	24	0	121
d				+	[5] A(T1)CL-3	−	0.05	0.00004	24	0	28
27 a	Adriamycin	23214-92-8	[+m?]	+	[4] CHO	+	0.1	0.00018	5	0	19
b				+	[1] Hum lymph	−	5.4	0.01	1	0	113
c				+	[1] Hum lymph	−	0.06	0.00011	48	0	198
d				+	[1] Hum lymph	−	0.02	0.000037	24	0	200
e				+	[1] Hum lymph	−	0.1	0.00018	4	48	318
f				+	[1] Hum lymph	−	0.05	0.00009	4	24	317
g				+	[2] Hum fibro	−	0.01	0.000018	1	5	200
h				+	[4] CHO	−	0.1	0.00018	5	0	19
i				+	[4] CHO	−	0.1	0.00018	0.5	7	103
28	Aflatoxin B1	1162-65-8	[+m+]	+	[4] V79	+	0.5	0.0016	1	26	23
30	Alachlor	15972-60-8	[M]	+	[1] Hum lymph	−	2	0.0074	24	0	88
88 a	Azathioprine	446-86-6	[+m+]	+	[1] Hum lymph	−	50	0.2	24	0	210
b				+	[1] Hum lymph	−	23	0.083	24	0	313
c				−	[1] Hum lymph	−	40	0.14	24	0	7
92 a	Barbital	57-44-3	[−M]	+	[4] CHL	−	2000	11	48	0	122
b				−	[4] DON	−	1470	8	26	0	1
98 a	Benzene	71-43-2	[−$\overset{M}{m}$+]	+	[1] Hum lymph	+	9	0.11	3	25	110
b				+	[4] CHL	+	550	7.6	6	18	123
c				+	[4] CHO	+	200	2.6	3	15	218
d				+	[1] Hum lymph	−	16	0.2	53	0	183
e				+	[1] Hum lymph	−	9	0.11	3	25	110
f				+	[1] Hum lymph	−	86	1.1	72	0	148
g				−	[4] CHO	+	5000	64	2	10	92

98 h				−	[4] CHO	+	936	12	1	19	195
i				−	[4] CH1-L	−	250	3.2	36	0	54
j				−	[4] CHO	−	1600	20.5	10	3	92
k				−	[4] CHL	−	4400	56.3	6	18	123
l				−	[4] CHO	−	800	10.3	3	15	218
m				−	[4] CHO	−	936	12	1	19	195
n				−	[6] RL4	−	1000	12.8	24	24	229
100 a	Benzidine	92-87-5	[+m+]	+	[1] Hum lymph	+	200	1.1	3	24	12
b				+	[4] CHO	+	3330	18	1	12	191
c				+	[1] Hum lymph	−	400	2.2	3	24	12
d				+	[4] CH1-L	−	8	0.043	36	0	152
e				+	[4] CHO	−	3330	18	1	12	191
f				+	[6] RL4	−	2.5	0.014	24	0	230
g				+	[6] RL4	−	40	0.22	24	0	170
101 a	Benzo[a]pyrene	50-32-8	[+m+]	+	[2] W1-38	+	5	0.02	1	24	328
b				+	[4] CHO	+	13	0.05	2	20	329
c				+	[4] CHO	+	3330	13	1	12	191
d				+	[4] CHL	+	10	0.04	6	18	121
e				−	[4] V79-4	+	1	0.004	24	0	227
f				−	[2] W1-38	−	10	0.04	24	0	328
g				−	[4] CHO	−	130	0.5	2	20	329
h				−	[4] CHO	−	5000	20	1	12	191
i				−	[4] CHL	−	40	0.16	6	18	121
115	1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU)	154-93-8	[$\overset{M}{m}$]	+	[4] CHL	−	3.8	0.018	24	0	121
130 a	Bromodichloromethane	75-27-4	[+m]	+	[4] CHL	+	240	1.5	3	21	121
b				−	[4] CHL	−	490	3	48	0	121
133 a	Busulfan (myleran)	55-98-1	[+$\overset{M}{m}$?]	+	[1] Hum lymph	−	10	0.04	72	0	81
b				+	[1] Hum lymph	−	1	0.004	72	0	108
c				+	[4] CHL	−	2000	8.1	48	0	121
192 a	Chlorambucil	305-03-3	[+M+]	+	[1] Hum lymph	−	0.25	0.0008	48	0	237
b				+	[1] Hum lymph	−	0.5	0.0016	72	0	275
247	Cyclohexylamine	108-91-8	[−M−]	+	[8] PTK1	−	50	0.5	24	48	91
248 a	Cyclophosphamide	50-18-0	[+$\overset{M}{m}$+]	+	[1] B35M	+	12.5	0.048	48	0	116
b				+	[1] Hum lymph	+	23	0.09	3	30	166
c				+	[4] CHO	+	50	0.19	5	0	19
d				+	[4] CHO	+	26	0.1	5	0	16
e				+	[5] A(T1)CL-3	+	50	0.2	1	24	30
f				+	[7] C3H10T1/2	+	50	0.2	1	24	30
g				+	[4] CHO	−	50	0.19	5	0	19
h				+	[4] CHL	−	3000	11	48	0	121
i				+	[6] RL1	−	1000	3.8	24	0	61
j				−	[4] CHO	−	104	0.4	5	0	16

TABLE 3 (continued)

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration (μg/ml)	Concentration (mM)	TRT (h)	REC (h)	Ref.
295	Diglycidylaniline	2095-06-9	[m]	+	[4] CHO-K1	–	8	0.039	24	0	258
305 a	4-Dimethylaminoazo-benzene	60-11-7	[+m+]	+	[4] CH1-L	–	25	0.11	36	0	152
b				–	[4] CHO	+	12500	55	1	12	191
c				–	[4] CHO	–	12500	55	1	12	191
d				–	[6] RL4	–	50	0.22	24	0	230
e				–	[6] RL4	–	80	0.36	24	0	170
308 a	7,12-Dimethylbenz[*a*]-anthracene	57-97-6	[+M m+]	+	[4] V79-4	+	1	0.0039	24	0	227
b				+	[4] CHL	+	50	0.2	6	18	121
c				+	[6] RL1	–	12.5	0.049	24	0	61
d				–	[4] CHL	–	200	0.78	6	18	121
309 a	Dimethylcarbamoyl-chloride	79-44-7	[+m+]	+	[4] CHO	+	0.0167 μl/ml		1	12	191
b				+	[4] CHO	–	0.1 μl/ml		1	12	191
312 a	Dimethylnitrosamine	62-75-9	[+m+]	+	[1] Hum lymph	+	3750	50	0.75	24	31
b				+	[4] CHO	+	2000	27	1	24	194
c				+	[4] CHL	+	500	6.7	6	18	121
d				+	[5] S H Fibro	+	7400	100	3	24	203
e				–	[4] CHL	–	2000	27	48	0	122
f				–	[5] S H Fibro	–	7400	100	3	24	203
g				–	[6] Rat lymph	–	6.7	0.09	24	0	162
331 a	Epichlorohydrin	106-89-8	[+M+]	+	[4] CHO	+	40	0.42	1	12	191
b				+	[1] Hum lymph	–	18.5	0.2	48	0	207
c				+	[4] CHO	–	120	1.3	1	12	191
d				+	[4] CHL	–	62.5	0.68	24	0	121
e				+	[6] RL1	–	5	0.054	24	0	61
355	Ethylene oxide	75-21-8	[+m ?]	+	[3] FL	–	220	5	1	48	226
360 a	Ethyl methanesulfonate	62-50-0	[+M m+]	+	[1] SN1029	–	0.003	0.000024	48	0	263
b				+	[1] HLCL	–	0.03	0.00024	48	0	263
c				+	[4] CHL	–	500	4	24	0	121
364 a	*N*-Ethyl-*N*-nitrosourea	759-73-9	[+M+]	+	[2] Hum fibro	–	11.7	0.1	1	5	249
b				+	[4] CHL	–	62.5	0.53	48	0	122
378 a	Fenitrothion	122-14-5	[+M–]	+	[4] CHL	+	50	0.18	3	21	121
b				+	[4] CHL	–	100	0.36	24	0	121
388 a	5-Fluorouracil	51-21-8	[+m ?]	+	[4] CHO	–	100	0.77	24	0	168
b				+	[4] CHL	–	1	0.008	24	22	333
c				+	[4] CHL	–	3.125	0.024	48	0	121

393 a	Furylfuramide (AF-2)	3688-53-7	[+m+]	+	[4] CHL	+	80	0.32	3	21	121
b				+	[1] Hum lymph	−	7.4	0.03	24	0	296
c				+	[4] CHL	−	5	0.02	48	0	121
d				+	[7] FM3A	−	7.4	0.03	48	0	310
433 a	Hexamethylphosphor-amide (HMPA)	680-31-9	[−m+]	+	[1] Hum lymph	+	100	0.56	3	25	9
b				+	[1] Hum lymph	+	500	2.8	3	25	110
c				+	[1] Hum lymph	−	100	0.56	3	25	9
d				+	[1] Hum lymph	−	500	2.8	3	25	110
e				+	[4] CH1-L	−	2	0.01	36	0	54
f				+	[4] CHL	−	6000	33.5	48	0	123
g				−	[4] CHO	+	340	1.9	1	12	191
h				−	[4] CHO	+	5000	28	2	10	92
i				−	[4] CHO	+	10000	56	3	15	218
j				−	[4] CHO	−	340	1.9	1	12	191
k				−	[4] CHO	−	5000	28	10	3	92
l				−	[4] CHO	−	10000	56	3	15	218
m				−	[6] RL4	−	2000	11.2	24	24	229
440	Hycanthone	3105-97-3	[+m?]	+	[5] A(T1)CL-3	−	1	0.0028	24	0	28
441	Hycanthone methane-sulfonate	23255-93-8	[+m?]	+	[1] Hum lymph	−	5	0.011	24	0	236
443 a	Hydralazine	304-20-1	[M]	+	[4] CHL	−	3.75	0.019	48	0	121
b	hydrochlorine			+	[7] FM3A	−	3.9	0.02	48	0	147
460	*N*-Hydroxyurethane	589-41-3	[+m+]	+	[1] Hum lymph	−	105	1	48	0	214
486 a	Kaempferol	520-18-3	[+m]	+	[4] CHO-AT3-2	+	35	0.12	15	0	45
b				+	[4] CHO-AT3-2	−	25	0.09	15	0	45
506 a	Malathion	121-75-5	[−M−]	+	[4] CHL	+	100	0.3	3	21	121
b				+	[1] Hum lymph	−	10	0.03	24	0	322
c				+	[4] CHL	−	60	0.18	48	0	121
d				−	[1] B411-4	−	100	0.3	50	0	115
510	Mancozeb	8018-01-7	[M]	+	[1] Hum lymph	−	4		24	0	88
522 a	6-Mercaptopurine	50-44-2	[+m?]	+	[1] Hum lymph	−	0.01	0.00007	52	0	190
b				+	[4] CHO	−	10	0.066	24	0	168
c				+	[4] CHL	−	2.5	0.016	24	22	333
d				+	[4] CHL	−	0.75	0.0049	48	0	121
e				−	[1] Hum lymph	−	5	0.03	24	0	210
f				−	[5] A(T1)-CL-3	−	100	0.66	24	0	28
530 a	Methotrexate	59-05-2	[−m?]	+	[4] CHO	+	5	0.011	5	0	19
b				+	[4] CHO	−	1	0.0022	24	0	168
c				+	[4] CHO	−	5	0.011	5	0	19
d				+	[5] A(T1)CL-3	−	1	0.0022	24	0	28

TABLE 3 (continued)

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration (μg/ml)	Concentration (mM)	TRT (h)	REC (h)	Ref.
572 a	Methyl methanesulfonate	66-27-3	[+$\overset{M}{m}$+]	+	[1] Hum lymph	–	22	0.2	24	0	42
b				+	[1] SN1029	–	0.003	0.000027	48	0	263
c				+	[1] HLCL	–	0.003	0.000027	48	0	263
d				+	[4] V79	–	27.5	0.25	24	24	141
e				+	[4] CHL	–	10	0.09	24	0	121
575 a	*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine	70-25-7	[+m+]	+	[1] Hum lymph	–	2.9	0.02	1	0	113
b				+	[1] SN1029	–	0.003	0.00002	48	0	263
c				+	[1] HLCL	–	0.003	0.00002	48	0	263
d				+	[2] Hum fibro	–	7.4	0.05	3	20	323
e				+	[2] W1-38	–	1	0.0068	24	0	328
f				+	[4] CHO	–	7.4	0.05	3	20	323
g				+	[4] CHO	–	3	0.02	2	24	112
h				+	[4] CHL	–	5	0.034	48	0	122
i				+	[4] DON, D-6	–	1.5	0.01	1	24	139
j				+	[4] V79-4	–	0.5	0.0034	24	0	227
k				+	[5] S H Fibro	–	0.3	0.002	3	24	120
l				+	[6] RL1	–	1	0.0068	24	0	61
584 a	*N*-Methyl-*N*-nitrosourea	684-93-5	[+M+]	+	[2] Hum Fibro	–	10	0.1	1	23	249
b				+	[4] DON	–	10	0.1	26	0	1
c				+	[4] CHL	–	25	0.24	48	0	122
d				+	[6] Rat lymph	–	93	0.9	24	0	162
604 a	Mitomycin C	50-07-7	[–$\overset{M}{m}$+]	+	[4] CHO	+	1	0.003	5	0	19
b				+	[1] Hum lymph	–	0.1	0.0003	24	0	50
c				+	[1] Hum lymph	–	0.1	0.0003	24	0	39
d				+	[1] SN1029	–	0.03	0.00009	48	0	263
e				+	[1] HLCL	–	0.03	0.00009	48	0	263
f				+	[4] CHO	–	1	0.003	5	0	19
g				+	[4] DON, D-6	–	0.33	0.001	1	24	139
h				+	[4] DON, D-6	–	0.017	0.00005	8	0	140
i				+	[4] CHL	–	0.04	0.00011	48	0	121
j				+	[8] MA61	–	0.6	0.0018	3	15	196
k				–	[1] Hum lymph	–	30	0.1	1	0	113
612 a	1-Naphthylamine	134-32-7	[+m?]	+	[4] CHO	+	17	0.12	1	12	191
b				–	[4] CHO	–	333	2.3	1	12	191
c				–	[4] CHL	–	60	0.42	48	0	122
613 a	2-Naphthylamine	91-59-8	[+m+]	+	[4] CHO	+	3.33	0.023	1	12	191
b				+	[4] CHO	–	5	0.035	1	12	191
632	Nitrofurantoin	67-20-9	[+m?]	+	[4] CHL	–	60	0.25	24	0	121

635 a	Nitrogen mustard	51-75-2	[+M m+]	+	[1] Hum lymph	–	0.02	0.0001	52	0	190
b				+	[4] CHO	–	15.6	0.1	2	0	104
644 a	4-Nitroquinoline	56-57-5	[+m+]	+	[2] W1-38	+	0.05	0.00025	1	24	328
b	1-oxide			+	[4] CHO	+	3	0.016	1	12	191
c				+	[1] SN1029	–	0.03	0.00016	48	0	263
d				+	[1] HLCL	–	0.03	0.00016	48	0	263
e				+	[2] Hum fibro	–	0.057	0.0003	3	20	323
f				+	[2] W1-38	–	0.03	0.00015	24	0	328
g				+	[4] CHO	–	2.3	0.012	1	12	191
h				+	[4] CHO	–	0.19	0.001	3	20	323
i				+	[4] CHL	–	0.2	0.001	24	0	122
j				+	[4] DON, D-6	–	0.19	0.001	1	24	139
k				+	[4] DON	–	0.5	0.0026	4	21	279
l				+	[6] RL1	–	0.02	0.00011	24	0	61
m				+	[7] L5178Y	–	0.025	0.00013	9	0	280
n				+	[7] M10	–	0.025	0.00013	12	0	280
o				+	[7] Q31	–	0.025	0.00013	12	0	280
718	Potassium bromate	7758-01-2	[+M m+]	+	[4] CHL	–	62.5	0.37	24	0	121
721 a	Potassium chromate	7789-00-6	[+m+]	+	[1] Hum lymph	–	0.78	0.004	28	0	187
b				+	[4] DON	–	0.06	0.0012	48	0	149
c				+	[4] CHO	–	0.25	0.0048	30	0	158
d				+	[7] FM3A	–	0.3	0.002	48	0	308
725 a	Potassium dichromate	7778-50-9	[+M]	+	[1] Hum lymph	–	0.073	0.00025	70	0	271
b				+	[1] Hum lymph	–	0.15	0.0005	28	0	187
c				+	[4] CHO	–	0.3	0.001	32	0	157
d				+	[4] CHO	–	0.1	0.002	32	0	316
e				+	[4] CHO	–	0.1	0.0019	30	0	158
f				+	[4] V79-4	–	0.35	0.001	24	24	199
g				+	[5] S H Fibro	–	0.1	0.0003	24	0	298
h				+	[5] S H Fibro	–	0.1	0.00034	24	24	299
i				+	[7] FM3A	–	0.02	0.00064	24	0	308
735 a	Propanesultone	1120-71-4	[+M+]	+	[4] DON	–	12	0.1	26	0	1
b				+	[4] CHL	–	62.5	0.5	24	0	122
771 a	Quercetin	117-39-5	[+m ?]	+	[4] CHL	+	200	0.66	3	21	121
b				+	[4] CHO-AT3-2	–	6	0.02	15	0	45
c				+	[4] CHO	–	100	0.33	3	20	277
d				+	[4] CHL	–	60	0.2	48	0	121
773	Quinacrine mustard	64046-79-3	[+m]	+	[4] CHO	–	9.4	0.02	2	24	112
780 a	Rhodamine B	81-88-9	[+M+]	+	[4] CHO	–	10	0.02	5	0	15
b				+	[4] CHL	–	60	0.13	48	0	121
c				+	[8] Munt fibro	–	4	0.0083	24	0	160
818	Sodium chlorite	7758-19-2	[+m]	+	[4] CHL	–	20	0.22	24	0	121

TABLE 3 (continued)

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration (μg/ml)	Concentration (mM)	TRT (h)	REC (h)	Ref.
858	Streptonigrin	3930-19-6	[M]	+	[1] Hum lymph	−	0.001	0.000002	6	0	52
878 a	3,3′,5,5′-Tetra-	54827-17-7	[−m]	−	[4] CHO	+	5000	21	1	12	191
b	methylbenzidine			−	[4] CHO	−	5000	21	1	12	191
890 a	Thio-TEPA	52-24-4	[+m+]	+	[1] Hum lymph	−	1	0.0053	32	0	36
b				+	[4] CHO	−	10	0.053	24	0	168
c				+	[4] CHL	−	0.94	0.0049	48	0	121
d				+	[5] A(T1)CL-3	−	1	0.0053	24	0	28
898	Tolbutamide	64-77-7	[−m−]	−	[4] CHL	−	500	1.8	48	0	121
903 a	Trenimon	68-76-8	[+$\overset{M}{m}$+]	+	[1] Hum lymph	−	0.002	0.0000086	24	0	8
b				+	[2] Hum fibro	−	0.00002	0.000000086	24	0	8
c				+	[4] C H Fibro	−	0.00001	0.000000043	24	0	8
d				+	[4] CHO	−	0.23	0.001	2	0	104
917 a	Triethylenemelamine	51-18-3	[+m+]	+	[3] HeLa	−	0.03	0.00015	24	0	334
b				+	[4] C H Fibro	−	0.1	0.00049	24	0	334
934 a	Urethane	51-79-6	[−m+]	+	[4] CHL	−	8000	90	48	0	122
b				−	[4] DON	−	7130	80	26	0	1
c				−	[4] V79-4	−	25	0.28	24	0	227
940	Vinblastine	865-21-4	[−m ?]	+	[4] DON	−	0.0039	0.000005	10	0	257
941 a	Vincristine	57-22-7	[−m ?]	−	[4] CHO	+	10	0.012	5	0	19
b				−	[4] CHO	−	1	0.0012	24	0	168
c				−	[4] CHO	−	10	0.012	5	0	19
d				−	[5] A(T1)-CL-3	−	0.05	0.000061	24	0	28

No.	The number of the test substance is that used by Ishidate et al. (1988) in their listing of all substances tested in vitro.
[ABC]	Results from other tests. A, Ames test (data from Ishidate et al., 1988); B, bone marrow test in rodents [m, micronuclei (Ishidate et al., 1988); M, metaphase aberrations (Thompson, 1986)]; C, carcinogenicity tests in rodents (Ishidate et al., 1988). +, Positive; −, negative; ?, unresolved; blank, untested.
Results in vitro	+, clearly positive; −, negative or not clearly positive.
Cell type	See Table 5.
MET ACT	−, without metabolic activation; +, with metabolic activation (usually S9 from rat liver).
Concentration	The lowest effective concentration (LEC) for positive (+) results or the maximum tested dose for negative (−) results.
TRT	Treatment time in h (H).
REC	Recovery time in h (H).
Ref.	Reference number as in Ishidate et al. (1988), in vitro clastogenicity data.

date and Sofuni, 1985). May require metabolic activation; Ashby et al. (1985) suggest that the genotoxic effects of HMPA may be *via* enzyme-mediated formation of formaldehyde which is clastogenic (Natarajan et al., 1983; Levy et al., 1983). Negative in RL4 (Priston and Dean, 1985) and CHO (Palitti et al., 1985) with or without activation, up to 11.2 and 56 mM respectively.

Verdict: Possibly detectable at < 10 mM in CHL with activation. RL4 and CHO appear unresponsive.

(f) Tetramethylbenzidine. Tested only in CHO (Natarajan and Van Kesteren-van Leeuwen, 1981); negative up to 21 mM with or without activation. One hour treatment only, sampled at 12 h.

Verdict: May be detectable at < 10 mM with a longer duration of treatment and later sampling to allow for mitotic delay. The in vivo data require confirmation.

(g) Urethane. Positive in CHL (Ishidate and Odashima, 1977) at 90 mM for 48 h without activation; inconclusive at 45 mM. With activation (6 h treatment) LEC = 225 mM (Ishidate, personal communication). Negative in DON up to 80 mM (Abe and Sasaki, 1977).

Verdict: Not detectable at 10 mM (or even 50 mM).

Conclusions

(a) Those assays that required doses in excess of 10 mM to detect clastogenicity would probably have given positive results at < 10 mM if current testing guidelines had been adopted. The exception is urethane which, even with rigorous testing, had an LEC value of 90 mM in CHL cells. There is an urgent need for LEC estimates for urethane in cell systems other than Chinese hamster. It should be noted also that urethane is non-mutagenic in Ames tests. [*Note added in proof:* Frölich and Würgler (1990) have recently shown in the somatic mutation and recombination test (SMART) in Drosophila that urethane requires to be metabolically activated probably *via* cytochrome P450-dependent enzyme activities.]

(b) In a few instances certain cell types appear to be totally unresponsive to particular chemicals even under optimal conditions. Reasons other than an insufficiency of test chemical must be sought to explain these observations.

(c) If this database can be taken as representative of clastogenic chemicals, we conclude that the great majority of chemicals which are clastogenic in vivo will be detected in vitro even if an upper dose limit of 10 mM is used, provided that a rigorous protocol is adopted. Of particular importance are the following:

(i) The use of optimal conditions for metabolic activation. A positive control which requires activation (e.g. cyclophosphamide) should always be included and used at a relatively low concentration (Preston et al., 1987) to test the efficiency of activation.

(ii) An adequate duration of treatment. When activation is used, the duration of treatment with the test chemical is limited by the toxicity of the S9 mix. The longest possible treatment time consistent with the non-toxicity and non-mutagenicity (Section 4) of the S9 mix alone should be used; e.g., at least 3 h in human lymphocytes and CHO cells. 1-h treatments are insufficient.

(iii) An appropriate sampling time to allow for cell-cycle delay.

(iv) The analysis of at least 200 cells per dose. With a typical background frequency of 2% aberrant cells, when 100 cells are scored there is less than a 40% chance of detecting even a quadrupling in aberration frequency. Increasing the sample size to 200 cells increases this power to about 70% (Margolin et al., 1986).

(d) The advantage of adopting an upper concentration limit of 10 mM for testing is that it will, rightly, exclude those chemicals which are only clastogenic at very high doses which are irrelevant to human exposure. This assumes that such chemicals show threshold responses, which appears to be the case for non-DNA reactive chemicals which are clastogenic at concentrations producing significant changes in the osmolality of the culture medium (section 2.1). The disadvantage is that a few chemicals which are potentially clastogenic in vivo will be 'missed'; on the basis of the Ishidate/Thompson database we conclude that the frequency will be low (1/66 = 1.5%). Lowering the cut-off concentration would certainly increase this frequency (Table 7) whereas raising the level to 20 mM would begin to pick up effects associated

TABLE 4

CHEMICALS WHICH ARE CLASTOGENIC IN VITRO BUT DO NOT INDUCE MICRONUCLEI OR METAPHASE ABERRATIONS IN RODENT BONE MARROW IN VIVO

Data from Ishidate et al. (1988) and Thompson (1986).

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration (μg/ml)	Concentration (mM)	TRT (h)	REC (h)	Ref.
5 a	Acetaminophen	103-90-2	[−M]	+	[1] Hum lymph	−	200	1.3	72	0	325
b				+	[4] CHO-K1	−	70	0.46	24	0	250
c				+	[4] CHL	−	30	0.2	48	0	121
17	Acid red (C I acid red 52)	3520-42-1	[−M]	+	[4] CHL	−	9000	15	48	0	121
18 a	Acridine	260-94-6	[−m−]	+	[4] CHL	+	200	1.1	3	21	173
b				+	[4] CHL	−	30	0.17	24	0	173
43	2-Amino-4-nitrophenol	99-57-0	[+m]	+	[4] CHL	−	15	0.1	48	0	121
48	4-Aminoquinoline-1-oxide	2508-86-3	[+M−]	+	[4] CHL	−	200	1.3	24	0	122
52	Ammonium chloride	12125-02-9	[−m]	+	[4] CHL	−	300	5.6	48	0	121
86	Auramine O	2465-27-2	[−m ?]	+	[4] CHO	−	6.4	0.02	5	0	15
159 a	Cadmium chloride	10108-64-2	[+m+]	+	[1] Hum lymph	−	0.3	0.0016	24	0	46
b				+	[4] CHO	−	5.5	0.001	36	0	63
c				+	[4] V79	−	1.8	0.01	2	22	211
d				+	[4] V79	−	1.8	0.01	2	22	213
e				−	[7] FM3A	−	5.9	0.032	48	0	308
162 a	Caffeine	58-08-2	[−m−]	+	[2] W1-38	+	2000	10	1	24	328
b				+	[1] Hum lymph	−	250	1.3	24	0	327
176 a	Captafol	2425-06-1	[+M]	+	[4] V79-CL-15	−	3.5	0.01	27	0	287
b				+	[4] CHL	−	4	0.011	24	0	121
c				−	[4] CHL	+	16	0.046	3	21	121
177 a	Captan	133-06-2	[+$^{m}_{M}$+]	+	[4] V79-CL-15	−	14	0.045	27	0	287
b				+	[4] CHL	−	7	0.023	24	0	121
c				−	[2] Hum fibro	−	4	0.013	24	0	286
227 a	Cinnamic aldehyde	104-55-2	[+m ?]	+	[4] B241	−	0.001	0.00001	24	24	138
b				+	[4] CHL	−	10	0.076	48	0	121
238	Cochineal	1260-17-9	[+M]	+	[4] CHL	−	12000	20	48	0	121
252 a	DDT	50-29-3	[−m ?]	+	[4] B14F28	−	8.1	0.023	4	0	167
b				−	[4] V79	−	45	0.13	24	24	141
258 a	2,4-Diaminoanisole	615-05-4	[+m+]	+	[4] CHO	+	50	0.36	1	16	57
b				+	[4] CHO	−	50	0.36	1	16	57

272 a	Dibromochloromethane	124-48-1	[+m]	+	[4] CHL	+	98	0.47	3	21	121
b				−	[4] CHL	−	610	2.9	48	0	121
285 a	Dichlorvos	62-73-7	[+$^{m}_{M}$?]	+	[1] Hum lymph	−	10	0.045	24	0	59
b				+	[4] V79-CL-15	−	110	0.5	27	0	287
c				+	[4] CHL	−	125	0.57	48	0	121
291 a	Diethylnitrosamine	55-18-5	[+$_{m}$+]	+	[4] CHL	+	3000	29	3	21	174
b				+	[4] CHO	+	10200	100	3	24	194
c				−	[4] CHL	−	3000	29	3	21	121
302	Dimethoate	60-51-5	[+M ?]	+	[4] CHL	−	500	2.2	48	0	121
325	Disodium glycyrrhizinate	71277-789-7	[−m]	+	[4] CHL	−	2000	2.3	48	0	121
339 a	Ethenzamide	938-73-8	[M]	+	[4] CHL	+	600	3.6	3	21	174
b				+	[4] CHL	−	500	3	48	0	121
341	Ethionamide	536-33-4	[−m ?]	+	[4] CHL	−	400	2.4	48	0	121
344	Ethylacetate	141-78-6	[−m ?]	+	[4] CHL	−	9000	100	48	0	121
373 a	Eugenol	97-53-0	[−m ?]	+	[4] CHL	−	125	0.76	48	0	121
b				−	[4] CHO	−	200	1.2	3	20	277
376	Fast green FCF (crude)	2353-45-9	[+m+]	+	[4] CHL	−	2000	2.5	48	0	121
379	Ferrous sulphate	7720-78-7	[m]	+	[4] CHL	−	1250	8.2	24	0	121
389 a	Formaldehyde	50-00-0	[+$^{M}_{m}$+]	+	[4] CHO	+	6	0.2	2	20	193
b				+	[2] Hum fibro	−	60	2	24	0	159
c				+	[4] CHO	−	6	0.2	2	20	193
420 a	Griseofulvin	126-07-8	[−m+]	+	[1] Hum lymph	−	40	0.11	60	0	155
b				+	[2] Hum eue	−	40	0.11	60	0	155
444 a	Hydrazine sulphate	10034-93-2	[+m+]	+	[4] CHO	+	158	1.2	1	12	191
b				+	[4] CHO	−	158	1.2	1	12	191
449 a	Hydrogen peroxide	7722-84-1	[+M]	+	[4] CHO-K1	−	3.4	0.1	3	24	297
b				+	[4] V79	−	3.4	0.1	3	24	297
c				+	[4] CHL	−	125	3.7	24	0	121
d				+	[5] SH Fibro	−	3.4	0.1	3	24	297
e				+	[7] Mus fibro	−	0.34	0.01	3	24	297
457	*N*-Hydroxymethyldimethyl phosphonopropionamide	20120-33-6	[−M]	+	[4] CHL	−	1000	4.4	24	0	121
459 a	Hydroxyurea	127-07-1	[−m ?]	+	[1] Hum lymph	−	25.3	0.333	48	0	214
b				−	[1] Hum lymph	−	37	0.1	1	0	113

TABLE 4 (continued)

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration (μg/ml)	Concentration (mM)	TRT (h)	REC (h)	Ref.
467	Iron and sodium succinato citrate		[−m]	+	[4] CHL	−	2000		24	0	121
482 a	Isoniazid	54-85-3	[+M]	+	[4] CHL	−	2000	15	24	0	121
b				+	[7] FM3A	−	440	3.2	48	0	147
c				−	[4] CHL	+	8000	58	3	21	174
491 a	Lacchaic acid A	60687-93-6	[−M]	+	[4] CHL	−	1500		48	0	121
b				−	[4] CHL	+	500		3	21	121
494 a	Lead acetate	301-04-2	[−m+]	+	[1] Hum lymph	−	3.3	0.01	24	0	26
b				−	[1] Hum lymph	−	330	1	3	48	86
c				−	[1] Hum lymph	−	330	1	72	0	254
501 a	D-Lysergic acid diethylamide (LSD)	50-37-3	[−m]	+	[4] CHL	+	1600	4.9	6	18	121
b				−	[4] CHL	−	62.5	0.19	48	0	121
507	Maleic anhydride	108-31-6	[$-^{M}_{m}$?]	+	[4] CHL	−	125	1.3	48	0	121
538	Methyl acrylate	96-33-3	[−m]	+	[4] CHL	−	7.5	0.087	24	0	121
559 a	3-Methylcholanthrene	56-49-5	[+m+]	+	[4] CHL	+	20	0.075	6	18	121
b				+	[6] RL1	−	2	0.0075	24	0	61
c				−	[4] V79-4	+	1	0.0037	24	0	227
d				−	[2] W1-38	−	10	0.037	24	0	328
e				−	[4] CHL	−	80	0.3	6	18	121
560 a	2-Methyl-4-dimethylamino-azobenzene	54-88-6	[+M]	+	[4] CHL	+	120	0.5	3	21	121
b				−	[4] DON	−	24	0.1	26	0	1
c				−	[4] CHL	−	30	0.13	48	0	122
633 a	Nitrofurazone	59-87-0	[$+^{M}_{m}$?]	+	[4] CHL	+	500	0.5	3	21	174
b				−	[4] CHL	−	10	0.05	48	0	122
642 a	4-Nitro-*O*-phenylenediamine	99-56-9	[+m−]	+	[4] CHL	−	30	0.2	48	0	122
b				+	[4] CHMP/E	−	25	0.16	24	0	144
651	*N*-Nitroso-ethylenethiourea	3715-92-2	[m]	+	[4] V79-CL-15	−	66	0.5	16	0	287
680	Perillaldehyde	2111-75-3	[−m]	+	[4] CHL	−	40	0.27	24	0	121
687 a	Phenobarbital	50-06-6	[+m?]	+	[4] CHO	+	500	2.2	2	10	92
b				+	[4] CHO	+	3500	15	1	19	195
c				+	[4] CH1-L	−	100	0.43	36	0	54
d				+	[4] CHO	−	1000	4.3	10	3	92
e				−	[4] CHO	+	400	1.7	1	25	240
f				−	[4] CHO	−	400	1.7	1	25	240
g				−	[4] CHL	−	2000	8.6	48	0	123
h				−	[4] CHO	−	3500	15	1	19	195
i				−	[6] RL4	−	1000	4.3	24	24	229

691 a	Phenylbutazone	50-33-9	[−m?]	+	[1] Hum lymph	−	98.6	0.32	24	0	331
b				+	[4] CHL	−	800	2.6	48	0	121
696 a	*P*-Phenylenediamine	106-50-3	[+m]	+	[4] CHL	+	500	4.6	3	21	121
b				−	[4] CHL	−	500	4.6	3	21	121
730 a	Potassium sorbate	24634-61-5	[−M]	+	[4] DON	−	3000	20	26	0	1
b				+	[4] CHL	−	4000	27	48	0	122
737 a	β-Propiolactone	57-57-8	[+m+]	+	[4] DON	−	72	1	26	0	1
b				+	[4] CHL	−	30	0.42	24	0	122
738 a	Propylene glycol	57-55-6	[−m]	+	[4] CHL	−	32000	420	48	0	121
b				−	[4] CHL	+	64000	840	3	21	121
742	Propyl gallate	121-79-9	[−M]	+	[4] CHL	−	20	0.094	24	0	121
772	Quinacrine dihydrochloride	69-05-6	[m]	+	[4] CHO	−	9.5	0.02	2	24	112
775 a	Quinoline	91-22-5	[+m+]	+	[4] CHL	+	300	2.3	3	21	174
b				−	[4] DON	−	129	1	26	0	1
c				−	[4] CHL	−	250	1.9	48	0	122
777 a	Resorcinol	108-46-3	[−m?]	+	[1] Hum lymph	−	20	0.18	24	0	56
b				+	[1] Hum lymph	−	80	0.73	24	0	255
c				+	[3] Hum amn	−	40	0.36	24	0	255
d				−	[4] CHO	+	1600	14	1	16	56
e				−	[2] Hum fibro	−	50	0.45	24	0	56
f				−	[4] CHO	−	400	3.6	16	0	56
778	Resorcinol diglycidyl ether	101-90-6	[m]	+	[4] CHO-K1	−	8	0.036	24	0	258
792 a	Safrole	94-59-7	[+m+]	+	[4] CHL	+	175	1.1	6	18	123
b				+	[4] CHO	+	83.3	0.5	3	15	218
c				−	[4] CHO	+	365	2.3	1	12	191
d				−	[4] CHO	+	50	0.31	2	10	92
e				−	[4] CHO	−	365	2.3	1	12	191
f				−	[4] CH1-L	−	60	0.37	36	0	54
g				−	[4] CHO	−	100	0.62	10	3	92
h				−	[4] CHL	−	150	0.92	6	18	123
i				−	[4] CHO	−	250	1.5	3	15	218
j				−	[6] RL4	−	100	0.62	24	24	229
824 a	Sodium dehydroacetate	4418-26-2	[−m]	+	[4] CHL	−	2000	5.3	48	0	122
b				−	[4] DON	−	19	1	26	0	1
829 a	Sodium fluoride	7681-49-4	[$-^{M}_{m}$?]	+	[2] JHU-1	−	20	0.48	24	0	302
b				+	[5] SH Fibro	−	100	2.4	28	0	305
c				−	[1] Hum lymph	−	130	3	48	0	290
831 a	Sodium hypochlorite	7681-52-9	[$+^{M}_{m}$]	+	[4] CHL	−	500	6.7	48	0	121
b				−	[4] CHL	+	125	1.7	3	21	121

TABLE 4 (continued)

No.	Test substance	CAS No.	[ABC]	Results in vitro	Cell type	MET ACT	Concentration (μg/ml)	Concentration (mM)	TRT (h)	REC (h)	Ref.
839	Sodium nitrite	7632-00-0	[+m?]	+	[4] CHL	−	500	7.2	48	0	122
860 a	Styrene oxide	96-09-3	[+m?]	+	[1] Hum lymph	−	80	0.67	8	0	165
b				+	[1] Hum lymph	−	12	0.1	48	0	75
c				+	[1] Hum lymph	−	24	0.2	48	0	207
d				+	[1] Hum lymph	−	80	0.67	8	0	164
e				+	[4] CHL	−	3.75	0.031	24	0	121
880 a	Theophylline	58-55-9	[−M]	+	[1] Hum lymph	−	500	2.8	24	0	327
b				+	[4] CHL	−	500	2.8	48	0	121
c				+	[7] FM3A	−	580	3.2	48	0	147
d				−	[4] CHL	+	2000	11	3	21	121
e				−	[1] Hum lymph	−	1800	10	72	0	295
904 a	Triamterene	396-01-0	[−M]	+	[4] CHL	−	3.75	0.015	48	0	121
b				−	[4] CHL	+	30	0.12	3	21	121
906 a	Tribromomethane	75-25-2	[+m?]	+	[4] CHL	+	116	0.46	3	21	121
b	(bromoform)			−	[4] CHL	−	440	1.7	48	0	121
907	Trichlorfon	52-68-6	[+M/m]	+	[4] CHL	−	125	0.49	48	0	121
923 a	Trisodium glycyrrhiz-	71277-78-6	[−m]	+	[4] CHL	+	4000	4.5	3	21	121
b	inate			+	[4] CHL	−	2000	2.2	48	0	121

See Footnote to Table 2 for explanations of headings and symbols except for M and m in the column headed [ABC]. In this Table M (metaphase aberrations) and m (micronuclei) refer to the tests performed, not to positive results obtained.

with high levels of osmolality. On balance, we recommend an upper concentration limit of 10 mM.

(e) If clastogenicity tests *are* performed at concentrations above 10 mM the osmolality of the culture medium should be measured. If there is a substantial increase (> 50 mOsm/kg) and the chemical nature of the test agent does not suggest DNA reactivity, clastogenesis as a consequence of the high osmolality of the culture medium should be suspected. For endpoints other than clastogenicity (Table 2) there are insufficient data to specify the osmolality levels at which these effects are likely to be seen.

2.2.2. LEC values in vitro for agents which are non-clastogenic in vivo

Table 4 lists, with LEC values, 68 chemicals which are clastogenic in vitro but not in vivo. These are also depicted in Fig. 2. Fig. 3 compares the LEC values of chemicals which are clastogenic in vivo and those which are not. There is a distinct

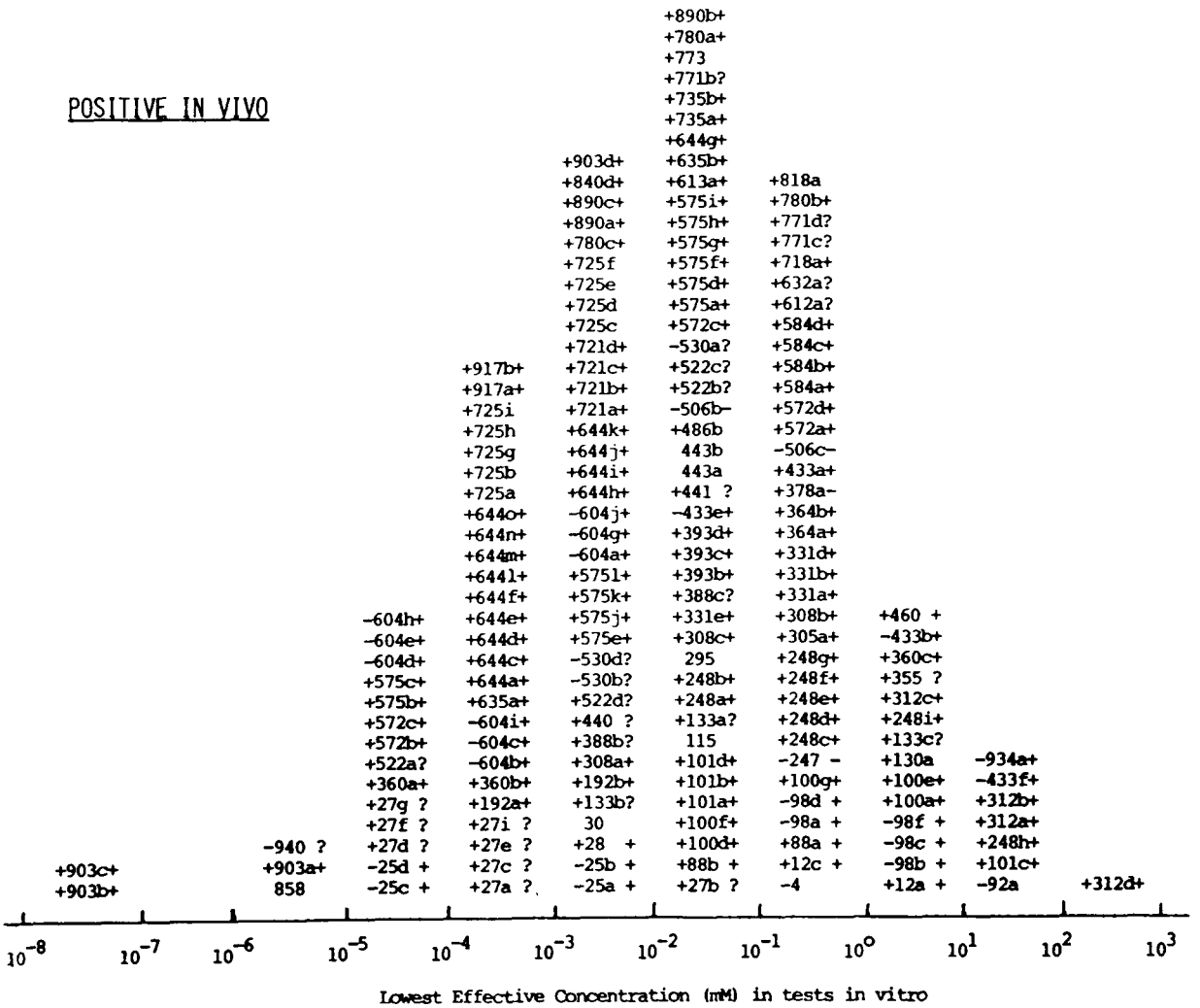

Fig. 1. Lowest effective concentrations in in vitro tests for those chemicals which induce micronuclei or metaphase aberrations in rodent bone marrow in vivo. Numbers refer to test substances in Table 3 which are clastogenic in vitro. Letters (a, b, c, etc.) refer to individual tests. Sign (+ or −) before the number indicates result of Ames test(s). Sign (+, − or ?) after the number indicates result of carcinogenicity test(s) in rodents. Where testing has been done with or without metabolic activation, the lower of the two LEC values has been plotted.

TABLE 5

DESCRIPTION OF THE CELLS USED IN STUDIES CITED IN TABLES 3 AND 4

Reproduced from Ishidate et al. (1988).

Abbreviation	Description
(*1*)	*Human lymphoid cells*
(1) B35M	A line of cells derived from a patient with Burkitt's lymphoma (Minowada et al., 1967)
(1) B411-4	A cell line derived from lymphocytes of a healthy person
(1) CCRF-CEM	A T-lymphoblastoid cell line derived from a patient with acute lymphoblastic leukemia (Foley et al., 1965)
(1) HLCL	Cell lines derived from human lymphocytes by the investigator and/or not otherwise identified
(1) Hum lymph	Peripheral lymphocytes from normal human donors cultured for a short time after stimulation with phytohemagglutinin
(1) P3J	A cell line derived from a patient with Burkitt's lymphoma (from Jiyoye, in Pulvertaft, 1964)
(1) RAJI	A cell line from a patient with Burkitt's lymphoma (Pulvertaft, 1964)
(1) RPMI-1788	A diploid cell line derived from lymphocytes of a normal human male donor (Huang, 1973)
(1) SN1029	A cell line derived from a patient with chronic lymphocytic leukemia (Shiraishi et al., 1979)
(*2*)	*Human fibroblast cells*
(2) Hum eue	A heteroploid fibroblast cell line
(2) Hum fibro	Fibroblast cultures from apparently normal humans (some fetal) which were established by the investigator, and/or not otherwise referenced
(2) JA	Human fibroblast cells cultured from the skin of a normal human donor
(2) JHU-1	Human fibroblast cells cultured from foreskin explants (Lin et al., 1980)
(2) MRC5	A diploid fibroblast cell strain derived from a normal male human donor (Jacobs, 1970)
(2) WI-38	A diploid fibroblast cell strain derived from a normal female human donor (Hayflick, 1965)
(*4*)	*Chinese hamster cells* (continued)
(4) CHO-K1	A clonal derivative of CHO cells which requires proline for growth (Kao and Puck, 1967)
(4) CHO-K1-A	An ouabain-resistant clonal derivative of the CHO-K1 cell line
(4) CHO-K1-BH4	A clonal derivative of CHO cells isolated by selection in aminopterin-containing medium (O'Neill et al., 1977)
(4) DON	A fibroblast cell line derived from lung tissue (Hsu and Zenzes, 1964)
(4) DON D6	A clonal derivative of the Don cell line
(4) HY	A line of fibroblast cells (Bauchinger and Schmid, 1972)
(4) V79	A line of fibroblast cells derived from lung tissue (Ford and Yerganian, 1958)
(4) V79-4	A clonal derivative of V79 cells (Chu et al., 1969)
(4) V79-CL-10	A clonal derivative of V79 cells
(4) V79-CL-15	A clonal derivative of V79 cells
(4) V79-E	A clonal derivative of V79 cells (Thust, 1979)
(*5*)	*Syrian hamster cells*
(5) A(T1)CL-3	A line of pseudodiploid fibrosarcoma cells (Benedict et al., 1975)
(5) SH fibro	Cultures of fibroblast cells established by the reporting investigator and/or otherwise not referenced
(*6*)	*Rat cells*
(6) MCT1	Cultures of cells derived from a chemically induced tumor in a Sprague–Dawley rat
(6) Rat fibro	Low passage cultures of rat fibroblasts established by the reporting investigator
(6) Rat lymph	Peripheral blood lymphocyte cultures established by the investigator
(6) RL1	An epithelioid cell line which was derived from rat liver and which retains enzymatic activity (Dean and Hodson-Walker, 1979)
(6) RL4	An epithelioid cell line which was derived from rat liver and which retains enzymatic activity

(3)	*Human cells, other types*
(3) CA-1	A tumor cell line derived from a human donor
(3) FL	An epithelioid cell line derived from human amnion and having a modal chromosome number of 63 (Fogh and Lund, 1957)
(3) HeLa	A line of epithelioid cells derived from a cervical carcinoma in a human donor (Gey et al., 1952)
(3) Hum amn	A culture of amniotic cells having a normal human karyotype
(3) VUP-1	A cell line from a malignant melanoma of the choroid in a human donor
(4)	*Chinese hamster cells*
(4) B14F28	A quasi-diploid line of fibroblasts derived from a Chinese hamster (see Yerganian and Leonard, 1961 for information on FAF28 cells, and Hsu et al., 1962 for notes on the B14FAF28 cells)
(4) B241	A near-diploid line of fibroblast cells
(4) CH Fibro	Chinese hamster fibroblasts cultured by the reporting investigator and/or not otherwise referenced
(4) CH1-L	A fibroblast cell line derived from the liver of a young male
(4) CHL	A clonal sub-line of fibroblasts derived from lung tissue (Koyama et al., 1970)
(4) CHMP/E	A near-diploid clonal derivative of a cell line established from the prostate
(4) CHO	A cell line derived from an ovary (Puck et al., 1958)
(4) CHO-A7	A clonal derivative of CHO that is deficient in arginase activity and requires ornithine or polyamines (Holtta and Pohjanpelto, 1982)
(4) CHO-AT3-2	A line derived from CHO in which the cells are heterozygous at both the aprt and tk loci (Adair et al., 1980)
(4) CHO-CL-10	A clonal derivative of CHO cells having a modal chromosome number of 23
(4) CHO-CL-H	A clonal derivative of CHO cells
(7)	*Mouse cells*
(7) C3H10T1/2	A cell line derived from the ventral prostate of C3H mouse embryos, then cloned (CL8) to yield a line of near-tetraploid cells (Reznikoff et al., 1973)
(7) FM3A	A line of cells derived from a C3H mouse mammary carcinoma which grows in suspension (Nakano, 1966)
(7) L cells	Cells taken from normal subcutaneous areolar and adipose tissue of an adult C3H/An mouse were cultured, then transformed in vitro by methylcholanthrene, cloned, and the clones tested for tumorigenic growth in vivo. Strain L cells are derived from one of these tumorigenic clones (Earle, 1942)
(7) L-929	A clonal derivative of L cells (Sanford et al., 1948)
(7) L5178Y	A line of murine leukemic lymphoblasts (See the reference cited as well as Fischer and Sartorelli, 1964)
(7) M10	A line of mutant L5178Y cells which is sensitive to ionizing radiation and 4-nitroquinoline-1-oxide (Sato and Hieda, 1979a)
(7) Mus fibro	Low passage cultures derived from the skin of newborn mice by the reporting investigator
(7) Q31	A line of mutant L5178Y cells which is sensitive to ultraviolet light and 4-nitroquinoline-1-oxide (Sato and Hieda, 1979b)
(8)	*Cells from other species*
(8) DH/SV40	Cultures of Djungarian hamster fibroblasts transformed by the SV40 virus
(8) MA61	Fibroblast cultures derived from the lung of the European vole, *Microtus agrestis*
(8) MUNT fibro	Fibroblast cultures derived from a male *Muntiacus muntjak* (Wurster and Benirschka, 1970)
(8) PTK-1	A cell line derived from the kidney of a rat kangaroo, *Potorous tridactylis*

For references see Ishidate et al. (1988). Where no reference is cited, the reference given in Tables 3 and 4 should be used.

TABLE 6

CHEMICALS WITH LOWEST EFFECTIVE CONCENTRATIONS (LEC) OF >10 mM FOR CLASTOGENESIS IN MAMMALIAN CELLS IN VITRO

Extracted from Ishidate et al. (1988).

No.	Test substance	CAS No.	Results	Cell type	MET ACT	LEC (μg/ml)	LEC (mM)	TRT (h)	REC (h)	A	m	C
9	Acetone	67-64-1	+	[4] CHL	–	40000	690	24	0	–		–
17	Acid red (C I acid red 52)	3520-42-1	+	[4] CHL	–	9000	15	48	0	–		
22	Acrylic acid	79-10-7	+	[4] CHL	–	750	10	48	0	–		
63	Aniline	62-53-3	+	[4] CHL	+	1000	11	3	21	–		
92	Barbital	57-44-3	+	[4] CHL	–	2000	11	48	0	–		
146	*N*-sec-Butyl-*N*-(methoxymethyl) nitrosamine	64005-63-6	+	[4] CHL	–	2000	14	48	0			
238	Cochineal	1260-17-9	+	[4] CHL	–	12000	20	48	0	+		
241	Creatinine	60-27-5	+	[4] CHL	–	10000	88	24	0			
289	Diethanolnitrosamine	1116-54-7	+	[4] CHL	–	5000	37	24	0	+		+
291a	Diethylnitrosamine	55-18-5	+	[4] CHL	+	3000	29	3	21	+	–	+
b			+	[4] CHO	+	10200	100	3	24			
343	8-Ethoxycaffeine	557-66-2	+	[4] CHO	–	4000	17	1	24			
344	Ethyl acetate	141-78-6	+	[4] CHL	–	9000	100	48	0	–	–	
354	Ethylene glycol	107-21-1	+	[4] CHL	–	26690	430	24	0	–		
361	*N*-Ethyl-*N'*-nitroguanidine	39197-62-1	+	[4] CHL	–	2000	15	48	0			
417	Glycodiazine	339-44-6	+	[4] CHL	–	8000	26	24	0			
434	*N*-Hexane	110-54-3	+	[4] CHL	–	5150	60	3	21	–		
526	Metformin hydrochloride	15537-72-1	+	[4] CHL	–	2000	12	48	0	–		
534	*N*-Methyl-*N*-acetylaminomethyl nitrosamine	59665-11-1	+	[4] CHL	–	2000	15	48	0			
537	*N*-Methyl-*N'*-acetylurea	623-59-6	+	[4] CHL	–	4000	34	48	0			

540	4-Methylamino-antipyrene	519-98-2	+	[4] CHL	–	5000	23	48	0			
574	*N*-Methyl-*N'*-nitroguanidine	4245-76-5	+	[4] CHL	–	3000	25	48	0	–		
623	Nicotinamide	98-92-0	+	[4] CHL	–	3000	25	48	0			
637	Nitroguanidine	556-88-7	+	[4] CHL	–	2000	19	24	0	–		
689	L-Phenylalanine	63-91-2	+	[4] CHL	–	2000	12	24	0	–		
719	Potassium bromide	7758-02-3	+	[4] CHL	–	4000	34	48	0			
730 a	Potassium sorbate	24634-61-5	+	[4] CHL	–	4000	27	48	0	–		
b			+	[4] DON	–	3000	20	26	0			
738	Propylene glycol	57-55-6	+	[4] CHL	–	32000	420	48	0	–	–	
791	Saccharin sodium	128-44-9	+	[4] CHL	–	8000	39	48	0	–		+
823	Sodium 5'-cytidilate		+	[4] CHL	–	30000	82	24	0	–		
827	Sodium D-tartrate	21106-15-0	+	[4] CHL	–	15000	87	48	0			
833	Sodium 5'-inosinate	4691-65-0	+	[4] CHL	–	10000	25	48	0			
838	Sodium nitrate	7631-99-4	+	[4] CHL	–	4000	47	24	0	–		
849	Sodium 5'-uridilate		+	[4] CHL	–	32000	87	24	0	–		
857	Streptomycin A	57-92-1	+	[7] FM3A	–	5800	10	48	0			
933 a	Urea	57-13-6	+	[1] Hum lymph	–	3000	50	72	0	–		
b			+	[4] CHL	–	12000	200	24	0			
934	Urethane	51-79-6	+	[4] CHL	–	8000	90	48	0	–	+	+
946	Xylitol	87-99-0	+	[4] CHL	–	16000	110	24	0	–		

No. The number of the test substance used by Ishidate et al. (1988) in their listing of all substances tested in vitro. Substances with LEC values of >10 mM in some cell types but <10 mM in others are not included.
Cell type See Table 5.
MET ACT + – with or without metabolic activation.
TRT duration of treatment (h).
REC recovery time (h).
A Ames test.
m micronucleus test in mice.
C carcinogenic in rodents.

TABLE 7

CHEMICALS WHOSE LEC VALUES FOR CLASTOGENICITY IN VITRO IN PARTICULAR CELL TYPES ARE >1, 5, 10, 20 or 50 mM

All chemicals induce micronuclei or metaphase aberrations in vivo in the bone marrow (see Table 3) and have been tested for clastogenicity in vitro.

Lowest effective concentration (mM)					Comments
>1.0	>5	>10	>20	>50	
2-Acetylamino-fluorine CHL+ (LEC = 2.2)					Difficult to activate? LEC = 0.13 mM in RL1
Barbital DON− (>8) CHL− (11)	Barbital DON− CHL−	Barbital CHL−			Not tested with activation or in other cell lines. Lowest effective dose (LED) in vivo = 330 mg/kg (Manna & Das, 1973)
Benzene CHO+ (2.6) CH1-L− (>3.2) CHL+ (7.6) RL4− (>12.8)	Benzene CHL+ RL4−	Benzene RL4−			LEC = 0.11 mM in HL. Probably requires metabolic activation. Floats on medium; cultures must be agitated during treatment. LED in vivo = 0.125 mg/kg (Heddle et al., 1983)
Benzidine HL+ (1.1) CHO+ (9.0)	Benzidine CHO+				Detectable at 0.22 mM in RL4
Busulphan CHL− (8.1)	Busulphan CHL− (8.1)				Detectable at <0.04 mM in HL
Cyclo-phosphamide RL1− (3.8)					Activation required. Positive in several cell types at <0.2 mM with activation.
Dimethylamino-azobenzene CHO+ (>55)	Dimethylamino-azobenzene CHO+	Dimethylamino-azobenzene CHO+	Dimethylamino-azobenzene CHO+	Dimethylamino-azobenzene CHO+	LEC = 0.11 mM in CH1-L. Difficult to activate. LED in vivo = 185 mg/kg (Heddle et al., 1983)

Dimethyl nitrosamine CHL+ (6.7) CHO+ (27.0) HL+ (50.0) SHF+ (100.0)	Dimethyl nitrosamine CHL+ CHO+ HL+ SHF+	Dimethyl nitrosamine CHO+ HL+ SHF+	Dimethyl nitrosamine CHO+ HL+ SHF+	Dimethyl nitrosamine SHF+	Difficult to activate? LED in vivo = 18.5 mg/kg (Heddle et al., 1983)
Ethyl methane-sulphonate CHL− (4.0)					LEC $< 2.4 \times 10^{-4}$ mM in other cell types
Ethylene oxide FL− (5.0)					
Hexamethyl-phosphoramide RL4− (> 11.2) CHL− (33.5) CHO+ (> 56)	Hexamethyl-phosphoramide RL4− CHL− CHO+	Hexamethyl-phosphoramide RL4− CHL− CHO+	Hexamethyl-phosphoramide CHL− CHO+	Hexamethyl-phosphoramide CHO+	LEC = 0.56 mM in HL and 0.01 mM in CH1-L. LED in vivo = 0.4 mg/kg.
Tetramethyl-benzidine CHO+ (> 21)	Tetramethyl-benzidine CHO+	Tetramethyl-benzidine CHO+	Tetramethyl-benzidine CHO+		Not tested in other cell lines. LED in vivo = 113 mg/kg (Heddle et al., 1983). In vivo data require confirmation
Tolbutamide CHL− (> 1.8)					Not tested in other cell lines
Urethane DON− (> 80) CHL− (90)	Urethane DON− CHL−	Urethane DON− CHL−	Urethane DON− CHL−	Urethane DON− CHL−	LEC = 225 mM in CHL+ (Ishidate personal communication). LED in vivo = 180 mg/kg (Heddle et al., 1983)

The + or − sign after the designation of the cell type indicates + or − metabolic activation. LEC values for each cell type are given in brackets in the first column. Where LEC values are given, for example, as '> 10', 10 mM was the highest dose used and was not clastogenic. Where testing has been done with and without metabolic activation, the lower of the two LEC values has been tabulated. Where more than one test has been done with the same cell type the lowest LEC value has been tabulated. Lowest effective doses (LED) in vivo (micronuclei or metaphase aberrations) are given under 'Comments' for those chemicals with LEC values of > 10 mM in vitro in one or more cell types. In Table 3 the LEC for cyclophosphamide in CHL− is 11.0 mM; when tested with activation LEC = 0.04 mM (Ishidate, personal communication).

difference in LEC values, attesting to the importance of the concentration dependency of in vitro clastogenesis in determining the probability of in vivo activity. The modal LEC for in vivo positives (10^{-1} to 10^{-2} mM) is approximately an order of magnitude less than for in vivo negatives (10^{-1} to 1.0 mM) and chemicals which are clastogenic in vitro at $< 10^{-2}$ mM (more so at $< 10^{-3}$ mM) have a high probability of being clastogenic in vivo. However, as we have seen (Fig. 1, Table 7) the converse is not the case; chemicals with high in vitro LEC values are not necessarily nonclastogenic in vivo.

The database in Table 4 is not sufficiently extensive to determine whether in vivo negative chemicals are detected in some cell types more than in others i.e. whether some cell systems are 'over-sensitive'. For the cell types most commonly used for in vitro testing, Chinese hamster fibroblasts and human lymphocytes, only 8 in vivo negative chemicals have been tested in both (Nos. 5, 159, 285, 389, 691, 777, 860 and 880 in Table 4). All but one of these were clastogenic in both systems, the exception being resorcinol (number 777) which was positive only in lymphocytes (LEC 0.18 mM).

Of the 68 chemicals that are clastogenic in vitro but not in vivo (Fig. 2) only a relatively small proportion ($10/68 = 14\%$) have LEC values of > 10 mM in one or more cell types (Table 8). Three of these 10 chemicals (caffeine, isoniazid and phenobarbital) had LEC values of < 10 mM in other cell types or in independent tests with the same cell type, and 2 chemicals (diethanolni-

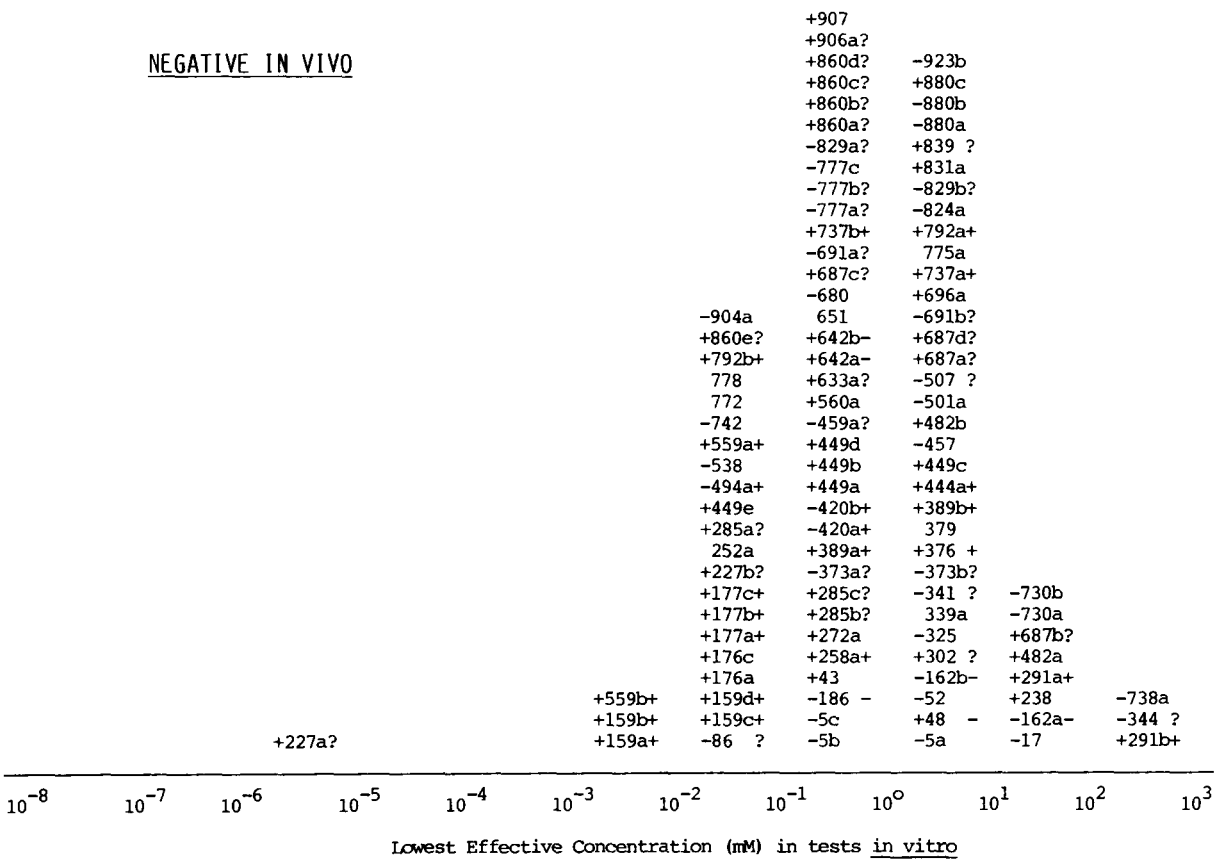

Fig. 2. Lowest effective concentrations in in vitro tests for those chemicals which do not induce micronuclei or metaphase aberrations in rodent bone marrow in vivo. Numbers refer to test substances in Table 4 which are clastogenic in vitro. See legend to Fig. 1 for other information.

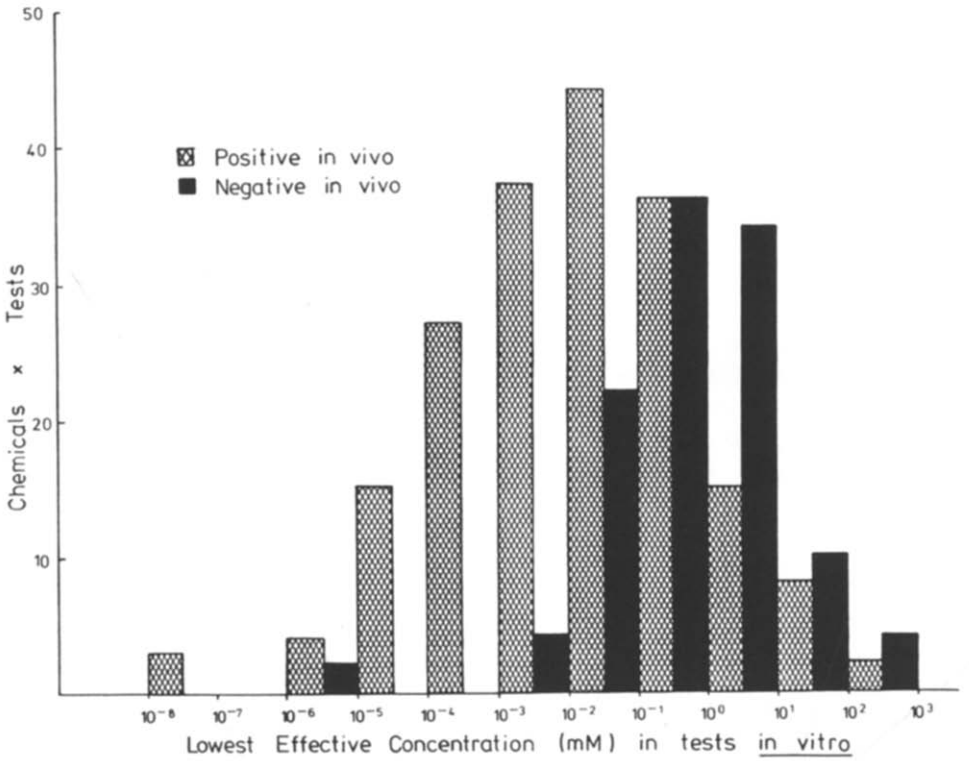

Fig. 3. Lowest effective concentration in in vitro tests for those chemicals which are clastogenic in vivo and those which are not. This diagram is simply a combination of data in Figs. 1 and 2 shown in histogram form to represent the number of tests in each LEC range.

trosamine and diethylnitrosamine) are DNA-reactive after metabolic activation. The clastogenicity of the remaining 5 chemicals (acid red, cochineal, ethyl acetate, potassium sorbate and propylene glycol) may be related to medium osmolality (although this was not measured) but the positive

TABLE 8

CHEMICALS WHICH ARE CLASTOGENIC IN VITRO AT >10 mM IN AT LEAST ONE CELL TYPE AND NON-CLASTOGENIC IN VIVO

(extracted from Table 4)

Number	Name	Cell type	LEC (mM)	Ames test	Carcinogen	Comment
17	Acid Red	CHL	15	–		
162	Caffeine	WI-38	10			LEC = 1.3 mM in human lymphocytes
238	Cochineal	CHL	20	+		Complex mixture
289	Diethanolnitrosamine	CHL	37	+	+	DNA-reactive
291	Diethylnitrosamine	CHL	29	+	+	DNA-reactive
		CHO	100			
344	Ethylacetate	CHL	100	–		Non DNA-reactive
482	Isoniazid	CHL	15			LEC = 3.2 mM in FM3A cells
687	Phenobarbital	CHO	15			LEC <10 mM in other tests with CHO and in CH1-L
730	Potassium sorbate	DON	20	–		Non DNA-reactive
		CHL	27			
738	Propylene glycol	CHL	420	–		Non DNA-reactive

Ames test with cochineal (Ishidate, 1988) suggests some other mechanism, and the LEC of 15 mM for acid red is probably too low to induce osmotic stress (see Table 2). Thus, only a small part (perhaps around 5%; 3/68) of the discrepancy between in vivo and in vitro results can possibly be attributed to osmotic effects in vitro. Other reasons must be sought for this large discrepancy.

A further possible contribution to this discrepancy which relates to the concentrations used for in vitro testing is that even when clastogenesis is detected at relatively low concentrations in vitro these concentrations may not be achievable in vivo because they exceed the tolerance of the test animals. The clastogenicity of fluoride is an example. In extensive tests for the genotoxicity of fluoride in vitro, the lowest effective concentration (4.5 μg/ml) was for clastogenicity in human fibroblasts exposed to sodium fluoride for 48 h; a fairly clearcut threshold response was found at this concentration (Scott and Roberts, 1987). The 24 h oral LD_{50} dose in rats (30–50 mg/kg) gives a maximum plasma fluoride concentration of about 10 μg/ml which is maintained for less than 4 h; by 24 h the concentration is only about 1.0 μg/ml (DeLopez et al., 1976). Mice are more sensitive than rats to the acute lethal effects of fluoride (Lim et al., 1978) so maximum achievable plasma concentrations are likely to be lower. It is unlikely therefore that a clastogenic concentration of fluoride could be reached in a bone marrow metaphase or micronucleus test in mice; it is perhaps not surprising that such tests have been negative (e.g. Martin et al., 1983). If we assume that in vivo, in man, there are no cells which are more sensitive than the most sensitive cells in vitro then for this chemical there will be a large safety margin for human exposure because even in areas with fluoridated water supplies the steady-state plasma level is only around 0.05 μg/ml (Singer and Ophawagh, 1979), some 100 times lower than the LEC in cultured human fibroblasts. There appears to be a similar high safety margin for human exposure to caffeine which also shows a threshold response for clastogenicity in vitro (Kihlman, 1977).

In spite of the fluoride and caffeine examples it is not always wise to disregard a positive in vitro response on the basis of dose dependency, using the argument that 'This concentration could never be achieved in vitro', because:

(a) it would be necessary to demonstrate a true threshold response. For DNA-reactive agents there are very few examples of concentration thresholds for genotoxicity and indeed '... the central mechanism of chemical attack on DNA should in principle be a non-threshold process...' (Ehling et al., 1983). On the other hand, some genotoxic effects which result from mechanisms not involving direct DNA interaction might be expected to be of the threshold type. For example, chemicals which inhibit the enzymes involved in DNA synthesis and DNA repair may be clastogenic. If the inhibition is not rate-limiting at low concentrations, there being an excess of enzyme, the dose–response will be of the threshold type. Threshold responses may also occur when clastogenesis results from cellular energy depletion or from the production of active oxygen species when concentrations necessary to overwhelm cellular antioxidant defences may be required before DNA damage occurs. Examples of 'indirect' genotoxicity including those that might be expected to show threshold responses are given in Table 9. The extent of the dose–response studies required to satisfactorily demonstrate a threshold response for a particular chemical is very considerable (see Scott and Roberts, 1987). Confidence in the existence of a threshold must come primarily from an understanding of the mechanisms involved.

(b) the sensitivity of cultured mammalian cells may not adequately reflect the sensitivity of cells in vivo particularly when metabolic activation is required. The insensitivity of some in vitro tests relative to responses in vivo is clearly seen in Table 7. In addition, Ishidate et al. (1988) cite some striking examples of differences in sensitivity to particular chemicals between different types of cultured cells and between different protocols using the same cell type (see also Tables 3, 4 and 7 in this Report).

In the light of these considerations, from the viewpoint of the concentration dependency of in vitro clastogenesis it would appear a wise precaution to follow up all in vitro positive results, regardless of the doses required, with an in vivo assay. Possible exceptions are non-DNA-reactive agents which appear to be clastogenic only at concentrations > 10 mM and where there is a

TABLE 9

INDIRECT MECHANISMS OF GENOTOXICITY [a]

Mechanisms	Cellular target	Examples
Enzyme inhibition	Enzymes of DNA synthesis	Hydroxyurea, fluorodeoxyuridine, aphidicolin, 2-deoxyadenosine (Kihlman and Natarajan, 1984), methotrexate (Benedict et al., 1977)
	Enzymes of DNA repair	Cytosine arabinoside, aphidicolin, 3-aminobenzamide (Kihlman and Natarajan, 1984), arabinofluranosyladenine (Nichols et al., 1980)
	Topoisomerase I	Camptothecin (Degrassi et al., 1989)
	Topoisomerase II	mAMSA [b] (Deaven et al., 1978), formaldehyde (Gaulden, 1987), VP-16 [c] (Palitti et al., 1990)
	Na^+/K^+ ATPase	Ouabain (Wangenheim and Bolcsfoldi, 1988)
Imbalance of DNA precursors	DNA precursors	DNA bases and nucleosides (MacPhee et al., 1988)
Energy depletion	Energy metabolism systems	Dinitrophenol (Mitchell and Simon-Reuss, 1952), cyanide (Kihlman, 1957; Umeda and Nishimura, 1979)
Production of active oxygen species	Oxygen and superoxide radicals	Paraquat (Nicotera et al., 1985), hydrogen peroxide (Tsuda, 1981)
Lipid peroxidation	Membranes	Phorbolmyristate acetate (Cerutti et al., 1983), asbestos (Cerutti et al., 1983), chromium chloride (Friedman et al., 1987)
Sulphydryl depletion	Sulphydryls	Diethylmaleate (Wangenheim and Bolcsfoldi, 1988)
Nuclease release from lysosomes	Lysosomes	*N*-Dodecylimidazole (Bradley et al., 1987), hypotonic medium (Nowak, 1987)
Inhibition of protein synthesis	Nuclear proteins	Cycloheximide (Wangenheim and Bolcsfoldi, 1988), Galloway et al. (unpublished data)
Protein denaturation	Nuclear proteins	Calcium hypochlorite (Ishidate et al., 1984), *N*-chloropiperidine (Ashby et al., 1987)
Ionic imbalance	Chromatin? Enzymes?	Ethylenediaminotetracetic acid, salts of saccharin, nitrilotriacetic acid, secalonic acid (Ashby, 1985), hypertonicity (Section 2.1)
?	?	Fluoride (refs. in Scott and Roberts, 1987)
?	?	Low pH (Section 5)

[a] The examples given are for clastogenesis except for ouabain, cycloheximide and diethylmaleate which induce 'mutations' at the thymidine kinase locus in L5178Y mouse lymphoma cells which may be point mutations or clastogenic events (Applegate and Hozier, 1987). Genotoxicity from hypertonic treatment (Table 2), low pH (Table 14) and fluoride (references in Scott and Roberts, 1987) also includes endpoints other than clastogenesis.

[b] m-AMSA, *N*-[4-(acridinylamino)-3-methoxyphenyl]methanesulphonamide.

[c] VP-16, an epipodophyllotoxin.

measured increase in the osmolality of the culture medium of > 50 mOsm/kg.

2.3. Extrapolation from in vitro genotoxicity for endpoints other than clastogenesis

Only for clastogenesis is there a sufficient database in vivo and in vitro to seriously address the question of upper concentration limits for in vitro testing which will detect all in vivo clastogens. Although the accumulated data on mutations at the HPRT locus in Chinese hamster cells is now considerable (Li et al., 1988) very few of these chemicals have been tested for HPRT mutagenesis in vivo. An alternative, indirect, approach has been to compare in vitro mutagenesis with rodent carcinogenicity but it is important to bear in mind that there are mechanisms of carcinogenicity that do not involve mutation or indeed any form of genotoxicity (Butterworth and Slaga, 1987). In comparing dose–response data for mutations at the thymidine kinase (TK) locus in mouse lymphoma cells with rodent carcinogenicity for the same chemicals, Wangenheim and Bolcsfoldi (1988) noted that virtually all rodent carcinogens were detected in vitro at less than 20 mM and recommend this as the upper concentration limit.

2.4. Summary and recommendations

There is increasing evidence of genotoxicity at very high concentrations as a consequence of the elevated osmolality of the culture medium. Such effects are unlikely to be of relevance to human exposure. To avoid this problem in clastogenicity tests an upper concentration limit of 10 mM has been suggested. However, some in vivo clastogens are only detectable in vitro at high concentrations; this may in part reflect the inadequacy of metabolic activation systems. Nevertheless, we conclude that if an upper concentration limit of 10 mM is used for testing, very few chemicals which are capable of in vivo clastogenesis in rodent bone marrow will be missed. We therefore recommend the use of an upper concentration of 10 mM in clastogenicity tests in vitro to avoid the artefacts associated with higher concentrations. Of the 50% of tested chemicals which are clastogenic in vitro but not in vivo probably less than 5% are clastogenic through osmolality effects. Other reasons must be sought for the large discrepancy between in vivo and in vitro responses. Even relatively low LECs in vitro may be higher than can be achieved in vivo with toxic chemicals. However, to be sure that an in vitro clastogen would be ineffective in vivo it is necessary to demonstrate a threshold response at a concentration well above that which could be achieved in vivo. Confidence in the existence of a true threshold must come primarily from an understanding of the mechanisms involved.

3. Genotoxicity and cytotoxicity

3.1. Introduction

Various guidelines for in vitro genotoxicity testing recommend inclusion of cytotoxic dose levels (see Table 1 for clastogenicity tests), with the intention of demonstrating a biological response in the system. This raises the question of whether genotoxic effects observed in vitro only at cytotoxic concentrations are relevant to the situation in vivo where cytotoxicity is likely to be less well tolerated and, in particular, to human experience where exposure concentrations will usually produce no, or minimal cytotoxic effects. If some degree of cytotoxicity *is* acceptable, can we define upper limits for in vitro test which would allow detection of all (or most) known in vivo genotoxins, as we have attempted for concentration dependency in Section 2? The aim would be to exclude chemicals which are genotoxic only above a certain level of cytotoxicity which could not be achieved in vivo.

3.2. Direct and indirect genotoxicity and cytotoxicity

The relationships between genotoxicity and cytotoxicity are complex. Apart from the wide spectrum of genotoxic endpoints (e.g. gene mutation, chromosome aberrations, sister-chromatid exchanges, DNA-strand breaks) there are many different in vitro assays of cytotoxicity. Some are measurements of cell death such as loss of colony-forming ability or cell lysis, whereas others are not necessarily associated with lethality, e.g. growth inhibition, cell-cycle delay, reduction in mitotic index and metabolic changes. These are dealt with in detail in Section 3.3.

RELATIONSHIPS BETWEEN DIRECT AND INDIRECT GENOTOXICITY AND CYTOTOXICITY

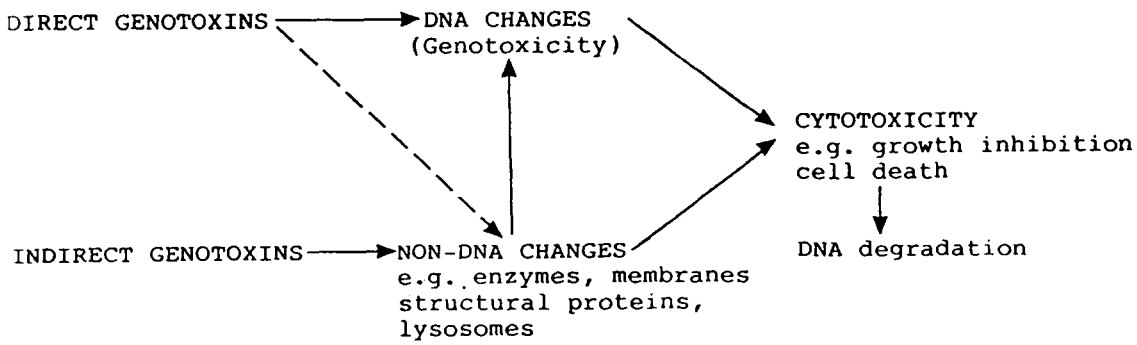

Fig. 4. Relationships between direct and indirect genotoxicity and cytotoxicity.

Genotoxic effects can result from *direct* interaction of chemicals with DNA through covalent binding or intercalation. These chemicals can induce a spectrum of DNA lesions that differ in their propensity to cause different genotoxic effects and in their susceptibility to repair (Swensen et al., 1980; Heflich et al., 1982; Liu-Lee et al., 1984; Natarajan et al., 1984). Gene mutations arise during the repair (misrepair) or replication (misreplication) of DNA carrying such lesions. More extreme alterations to the genome such as DNA-strand breakage and structural chromosome aberrations can also arise in this way. In addition, however, such gross changes can also be brought about without direct DNA interaction *via* a wide variety of *indirect* mechanisms (Table 9, Fig. 4). These include interference with the processes of DNA replication and repair; interaction with specific chromosomal non-histone proteins such as topoisomerase II, and with peripheral proteins; nuclease release from lysosomes; protein denaturation and the production of active oxygen species. Other methods of inducing these indirect effects by less well-understood mechanisms are through cellular energy depletion, pH changes (Section 5), tonicity changes (Section 2) and hyperthermia.

Since a significant proportion of chemicals that are genotoxic *via* indirect mechanisms probably exhibit threshold responses (Section 2.2.2), indirect genotoxins are likely to be of less importance than direct genotoxins from the point of view of human risk. Nevertheless, there are some indirect genotoxins that are active in vivo; for example, cycloheximide induces micronuclei in rodent bone marrow (Basic-Zaninov et al., 1987; Gulati et al., personal communication) and methotrexate is clastogenic in mice (Maier and Schmid, 1976) and in man (Jensen and Nyfors, 1979).

Agents which are genotoxic by either direct or indirect mechanisms are also cytotoxic. Cytotoxicity will result from the damage to the DNA itself (e.g. DNA-strand breakage and structural chromosome aberrations) and to other cellular targets (e.g. enzymes, membranes). For some agents the cytotoxicity will be expressed mainly through effects on DNA, whether induced directly (e.g. alkylating agents) or indirectly (e.g. DNA synthesis inhibitors) whereas for others the toxicity will be mediated mainly through damage to non-DNA targets (e.g. agents which induce lipid peroxidation, energy depletion, protein denaturation or ionic imbalance). A simplified scheme of the relationships between direct and indirect genotoxicity and cytotoxicity is shown in Fig. 4.

A mechanism of genotoxicity which can be considered as intermediate between direct and indirect mechanisms is when base analogues become incorporated into DNA at the time of DNA synthesis and induce chromosomal aberrations (e.g. bromodeoxyuridine; Hsu and Somers, 1961). For simplicity this mechanism is not shown in Fig. 4.

DNA changes that take place whilst cells are dying will not, of course, constitute a genetic hazard. DNA degradation in association with

necrosis in cultured mammalian cells has been reported after treatment with a variety of agents both genotoxic and non-genotoxic (Williams et al., 1974; Afanas'ev et al., 1986). A distinction must be made between induced DNA damage which is not necessarily cell lethal and is therefore potentially mutagenic (in the broadest sense) from DNA degradation which occurs in dying cells. This distinction is made in Fig. 4. The highly fragmented chromosomes in mitotic cells sometimes seen in clastogenicity assays at doses which induce significant cell death may be a manifestation of DNA degradation although it is important to note that several chemicals tested up to high levels of toxicity by Galloway et al. (1987b) were non-clastogenic, so chromosome aberrations are not an inevitable consequence of toxicity.

3.3. Assays of cytotoxicity

In genotoxicity tests various methods are used to assess the associated cytotoxicity induced by the test chemicals. As indicated above, some of these measure cell death, but other endpoints are also used.

The manifestations of cell death include:

(a) Loss of membrane integrity

This can be detected by using vital dyes and enzyme assays to detect membrane leakage (Roper and Drewinko, 1976). There are, however, problems of accurately quantifying cell death by this method because cells which are destined to die do not necessarily exhibit membrane damage immediately after treatment so the proportion of

TABLE 10

CYTOTOXICITY AND CHROMOSOME ABERRATIONS IN CHO CELLS TREATED WITH MITOMYCIN C OR ADRIAMYCIN

Armstrong et al., in preparation.

Chemical	Concentration (μM)	Sampling time										CFE% (7d)
		10 h					24 h					
		Cell count [a]	ATP/cell [b]	MI [c]	Aberrations [d]		Cell count	ATP/cell	MI	Aberrations		
					Abnormal cells (%)	per 100 cells				Abnormal cells (%)	per 100 cells	
Mitomycin C	0	100	100	15.9	3	3	100	100	10.5	4	4	100
	0.10	103	100	11.6	6	6	101	101	11.1	6	6	109
	0.25	106	105	7.4	11	13	93	106	13.8	14	15	96
	0.50	99	107	5.4	9	9	96	97	10.5	40	63	89
	0.75	105	98	7.5	16	20	78	127	16.2	74	140	75
	1.00	89	125	5.2	26	28	69	139	16.1	88	340	48
	2.00	99	112	4.2	31	38	57	177	10.0	100	530	10
	4.00	97	104	3.9	37	58	67	135	6.4	–	–	2
Adriamycin	0	100	100	20.9	2.0	2.0	100	100	13.3	3.0	3.5	100
	0.10	88	100	19.1	17.5	20.0	91	101	15.8	12.5	13.5	91
	0.25	80	106	8.9	70.0	114.0	82	99	16.7	20.5	35.0	92
	0.50	71	102	5.2	90.0	196.0	66	96	15.4	66.0	146.0	49
	0.75	71	98	1.6	100.0	580.0	55	91	10.7	82.0	240.0	9
	1.00	68	91	0.3	–	–	38	87	12.1	–	–	4
	2.00	66	93	0.4	–	–	33	91	11.1	–	–	0

Cells were treated for 3 h and sampled at 10 h and 24 h.

[a] Total viable cells as % of controls.

[b] ATP per cell as % of controls, measured by luminescence.

[c] Mitotic index based on 1000 cells scored.

[d] Aberration yields based on 200 cells scored except at high yields.

damaged cells will be different at different post-treatment sampling times. Also, membrane-damaged cells that lyse and disintegrate will not be included in the cell sample tested for viability; cell death will thus be underestimated unless a total cell count is compared to controls. Nevertheless, this method is useful when test cells are non-proliferating (as in some DNA damage/repair assays) or, for other reasons, cannot be evaluated for colony-forming efficiency.

(b) Loss of colony-forming efficiency (CFE)

This method is routinely used in mammalian cell mutation assays and sometimes in conjugation with other tests on clonogenic cells. It has the merit that, provided sufficient time elapses between treatment and observation to allow for colony-formation by surviving cells (usually 1 week or more), the surviving fraction remains constant thus giving a single numerical value.

Other assays, which do not necessarily measure cell death are:

(a) Reduction in cell numbers

Typically, cell counts in control and treated samples are made at 1 or 2 days after treatment. Reduction in cell number relative to controls will result both from cell lysis and, in proliferating cell populations, from a decreased growth rate. The ratio of cell numbers in control and tested samples will vary with sampling time (see Table 10). If cell counts are continued, in proliferating populations, well beyond the time when dead cells have been eliminated, a true estimate of cell killing can be obtained from back extrapolation of growth curves (Alexander and Mikulski, 1961) but this method is seldom used in genotoxicity tests because of its time-consuming nature.

(b) Mitotic index (MI)

In cytogenetic assays, doses are often chosen which induce some degree of inhibition of MI as an indication of biological responses. With typical control MI values of 5–10%, a 75–80% reduction in MI defines the upper concentration limit since at higher doses insufficient cells would be available for analysis. MI values can vary markedly at different times after treatment and may even increase above control levels (see Fig. 5 and Table 10) if chemicals induce partial synchronisation or cause arrest of cells in mitosis.

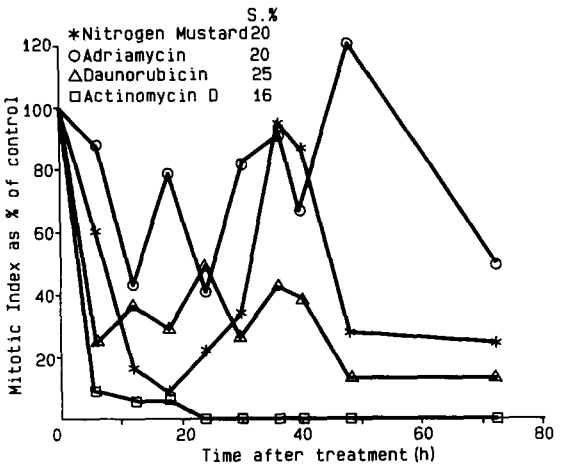

Fig. 5. Mitotic index as a percent of the untreated controls after treatment of asynchronous human fibroblasts with the clastogens, nitrogen mustard (0.2 μg/ml), adriamycin (0.25 μg/ml), daunorubicin (0.1 μg/ml) or actinomycin D (0.2 μg/ml) for 1 h. These doses induced a similar degree of loss of colony-forming efficiency which was assayed in parallel. S = survival (Data from Parkes and Scott, 1982)

Depression of the MI is usually a consequence of a reduced rate of cell proliferation (mitotic delay) but there may also be a contribution from cells which have permanently lost their proliferative capacity.

(c) Reduction in metabolic activity

This may be detected by measurements of ATP levels (e.g. Garrett et al., 1981; Garewal et al., 1986) or changes in dyes which require mitochondrial energy production (e.g. thiazolyl blue; Carmichael et al., 1987). Again, the extent of the reduction will vary as a function of time after treatment. The ATP content per cell can actually increase (Table 10) so that alterations are difficult to interpret. Suppression of ATP levels does not necessarily indicate cell death, as cells can recover from such depletion.

(d) Quantitative relationships between various endpoints of cytotoxicity

These relationships are complex (see Weisenthal et al., 1983; Roper and Drewinko, 1976). For example, for a series of chemicals, the dose levels

that gave a similar loss of CFE (75–85%) gave different degrees of mitotic inhibition for each chemical; the MI also varied with sampling time (Fig. 5).

Similarly, the ratio of acute killing (e.g., measured by trypan blue uptake at the end of a 3-h treatment) to delayed cell death (measured by CFE) varied amongst compounds. Cells treated with a direct DNA-damaging agent might show little sign of acute toxicity but could show marked suppression of colony-forming ability because of the lethal effects of DNA synthesis inhibition and of some types of chromosome aberrations. In contrast, an agent which killed cells rapidly, say by disruption of the cell membrane or energy production, would give acute toxicity, but the surviving cells when plated might or might not show good colony-forming efficiency, depending on the mechanism of killing. Perhaps signs of acute toxicity might raise the suspicion that any DNA damage subsequently detected was indirectly induced, but evidence for this is lacking. Information is badly needed on the dose–responses for cytotoxicity of agents that are indirectly genotoxic.

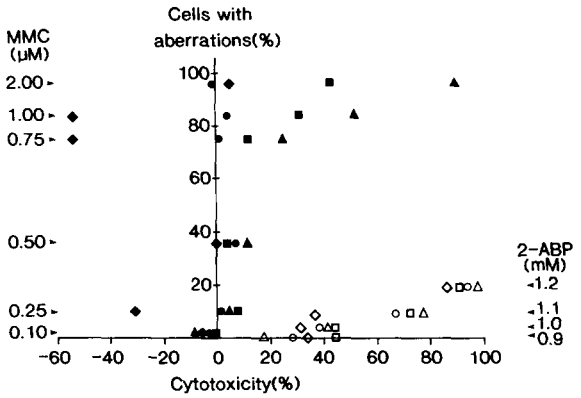

Fig. 6. Relationships between clastogenicity and cytotoxicity for mitomycin C (MMC) (solid symbols) and 2-aminobiphenyl (2-ABP) (open symbols) in CHO cells. Aberrations and cytotoxicity assayed at 24 h after treatment except CFE which was measured at 7 d. Cytotoxicity measured as percent reduction in cell counts (squares), ATP content (circles), mitotic index (diamonds) and CFE (triangles) relative to controls. Control aberration frequencies have been subtracted. MMC data are also given in Table 10. The concentration range for 2-ABP was from 0.7 to 1.2 mM. (Armstrong, Galloway et al., personal communication)

3.4. Quantitative relationships between genotoxicity and cytotoxicity

We have seen from the previous section (3.3) that, for most endpoints, the degree of cytotoxicity observed depends not only on the nature and concentration of a chemical but also on the sampling time after treatment. The most robust endpoint from this point of view is CFE which reaches a plateau level if sufficient time is allowed between the treatment and the assay. A similar problem of time-dependency is found for most genotoxic endpoints (e.g. chromosome aberrations, DNA damage) although, for gene mutations, a plateau level is reached when the full expression time is allowed. Since most genotoxicity tests utilise only one sampling time at which genotoxicity and cytotoxicity are assayed it follows that there are very few meaningful quantitative data relating these phenomena, the best being for gene mutation in relation to CFE. Nevertheless, it is evident that the ratio of genotoxicity to cytotoxicity varies among chemicals and that there are 'strong' and 'weak' genotoxins in this context as there are in relation to concentration (Section 2).

Relationships between genotoxicity and cytotoxicity will now be considered for the specific genotoxic endpoints; chromosome aberrations (CA), gene mutation and DNA-strand breakage.

3.5. Cytotoxicity in chromosome-aberration assays

There is a paucity of published quantitative data relating clastogenicity to cytotoxicity. Information on the latter is often omitted from publications because, frequently, the cytotoxicity of a test chemical is determined in pre-test, range-finding investigations with a wide range of concentrations, but not repeated in the final test at the concentrations examined for aberration induction.

Because there is no sizeable database we have been unable to examine the relationships between clastogenicity and cytotoxicity to the extent that was possible for clastogenicity versus concentration (Section 2). However, Armstrong, Galloway et al. (personal communication) have recently undertaken detailed studies on CA induction by a small number of chemicals which were known to be carcinogens, weak carcinogens or non-carcino-

gens in rodents. The induction of CA was measured at 10 and 24 h after treatment of Chinese hamster ovary (CHO) cells and concurrent measures of cytotoxicity were made in terms of cell counts, mitotic index, ATP content and CFE. We will use some of these data to illustrate the complexity of relationships between clastogenicity and cytotoxicity and the difficulty of using this information to predict in vivo response.

These investigations show that, typically, for a given chemical, clastogenicity/cytotoxicity relationships differ not only according to sampling time, but also according to the particular endpoint of cytotoxicity. For example (Table 10) at 10 h after mitomycin-C (MMC) treatment CA are observed without any reduction in cell count or ATP levels (e.g. at 0.25–0.50 μM), but with a substantial suppression in MI and some reduction in CFE (measured at 7 days). In contrast, at 24 h (Table 10, Fig. 6) very high aberration yields are seen at concentrations (e.g 0.75–1.00 μM) at which there is actually an *increase* in MI, following the suppression at 10 h. Cell counts are also reduced at 24 h. At high concentrations of MMC the clastogenicity/cytotoxicity ratios are very different for the different cytotoxic endpoints. For example, at 1.00 μM (see left hand column in Fig. 6), CFE was reduced by 52% compared with untreated cells, cell counts at 24 h by 32% and ATP levels by 4% whereas the MI at 24 h *increased* by 53% (shown as negative cytotoxicity on the abscissa). These ratios are, unusually, less variable with 2-aminobiphenyl (2-ABP), which is a weaker clastogen than MMC at any given level of cytotoxicity (Fig. 6). Incidentally, although there appears to be a threshold in the clastogenesis/cytotoxicity ratio for 2-ABP this was not confirmed in subsequent experiments using a series of sampling times for CA (Bean and Galloway, personal communication).

For the reasons given previously, the most meaningful comparison of clastogenicity and cytotoxicity is when cell killing, assayed as loss of CFE, is used as the measure of the latter. This relationship is shown in Fig. 7 for 7 chemicals studied by Armstrong, Galloway et al. The 24-h CA data are used for all chemicals other than adriamycin (Table 10) because for the other 6 chemicals the yields were higher at 24 h than at 10 h at most concentrations. It should be borne in mind, however, that accurate quantification of CA yields requires multiple sampling times when an asynchronous cell population is used since the patterns of yield versus time may differ for different chemicals (see Parkes and Scott, 1982). The yields at 24 h (or 10 h for adriamycin) are not necessarily the maximum frequencies induced by these chemicals; the peaks may be at different times. Bearing in mind this limitation it appears that these chemicals differ markedly in potency (Fig. 7) which, in this context, is the ratio of clastogenesis to cell death. Four of the chemicals [MMC, adriamycin (ADM), 2,4-diaminotoluene (2,4-DAT) and 2,6-diaminotoluene (2,6-DAT)] were very potent in that they induced substantial

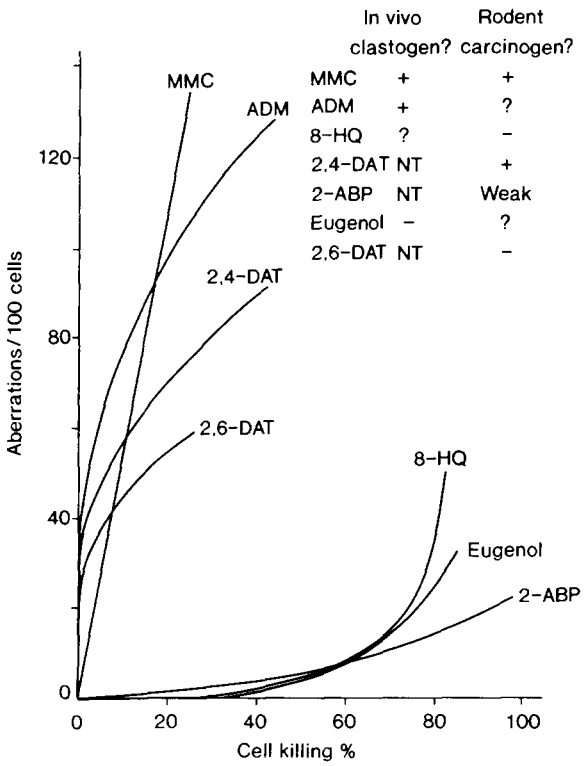

Fig. 7. Relationship between clastogenicity and cell killing (loss of CFE) in CHO cells exposed to mitomycin C (MMC, 0.25–4.00 μM), 2,4-diaminotoluene (2,4-DAT, 1.0–10.0 mM), 2-aminobiphenyl (2-ABP, +S9, 1.0–1.2 mM) or 2,6-diaminotoluene (2,6-DAT, 10–18 mM). Aberration yields are for cells fixed at 24 h and control frequencies have been subtracted. CFE was measured at 7 days. (Armstrong, Galloway et al., personal communication)

aberration frequencies (> 20 per 100 cells) at concentrations which produced little or no reduction in CFE. Clearly, CHO cells can tolerate a certain amount of structural chromosome damage without loss of viability. In contrast, the remaining 3 chemicals [8-hydroxyquinoline (8-HQ), eugenol and 2-aminobiphenyl (2-ABP)] were weak clastogens, inducing very low aberration frequencies at concentrations which reduced CFE by up to 50%.

Another notable difference among the clastogens was in the distribution of aberrations between cells. Whereas MMC and ADM produced a general increase in the proportions of cells with aberrations, only relatively low percentages of cells had aberrations after treatment with 2,4- and 2,6-DAT, or 8-hydroxyquinoline, but many of the cells that were damaged had multiple aberrations. This is important for several reasons. In testing chemicals for clastogenicity, many investigators report data only for the percentages of cells with aberrations. Clearly, since cells with multiple aberrations are rare in controls, the existence of such cells even when the percentage of aberrant cells is modest, is supporting evidence that the test compound is clastogenic. Also, a delayed harvest time is required to detect these cells (in the case of CHO cells, this was 24 h from the beginning of the 3-h treatment). Finally, this type of chromosome damage may be produced by indirect mechanisms (Fig. 4), perhaps in a subset of cells in a particular phase of the cell cycle. If this is the case then, at least for some chemicals, indirect clastogenesis is not necessarily associated with extreme cytotoxicity to the whole cell population; for example, the survival level at which cells with multiple aberrations were detected was 80% or more after treatment with 2,4- or 2,6-DAT. Cells suffering extreme chromosome damage are likely to lose their proliferative capacity but less severely damaged cells may not, and could constitute a long-term genetic hazard.

Can in vitro potency (clastogenicity with respect to cytotoxicity) be used to predict in vivo response? Of the 7 chemicals studied by Armstrong et al., only 4 have been tested for clastogenicity in rodent bone marrow. MMC is widely used as a positive control because of its clastogenicity in bone marrow cells of the mouse; it also induces aberrations in bone marrow cells of monkeys (Michelmann et al., 1978). ADM also induces aberrations in mouse bone marrow (e.g., Au and Hsu, 1980) and in human lymphocytes in vivo (Nevstad, 1978). MMC and ADM are both potent clastogens in CHO cells (Fig. 7). Eugenol induced aberrations in vitro at doses that reduced CFE and was reported negative in vivo, in a rat bone-marrow micronucleus assay (Maura et al., 1989). CA induction by 8-hydroxyquinoline (8-HQ) was only detectable at concentrations that caused a 75% reduction in CFE and a 50% reduction in cell count at 24 h. In vivo results with 8-HQ are marginal; McFee (1989) observed a small but non-significant increase in chromosome aberrations in mouse bone marrow, but Hamoud et al. (1989) detected small, but significant increases in bone-marrow micronuclei, particularly in normochromatic erythrocytes. 8-HQ is not a rodent carcinogen (Ashby and Tennant, 1988). If 8-HQ is truly clastogenic in vivo, it implies that weak clastogenicity in vitro detected at doses which are substantially cytotoxic cannot necessarily be discounted. Support for these conclusions comes from another study (Galloway et al., 1987b) in which at least 8 out of 24 clastogens were detectable in CHO cells only at dose levels that caused measurable cytotoxicity, assayed as a reduction in cell numbers ('reduced monolayer confluence') at 10–20 h after treatment, which was the time of harvesting for aberration analysis. In vivo data are available for only 2 of these 8 chemicals. Of these two, 2,4,5-T did not induce micronuclei in vivo (Davring and Hultgren, 1977; Jenssen and Ramel, 1980) whereas malathion reportedly induced aberrations in mouse bone marrow (Doulout et al., 1983) although this was not reproduced in another study using similar test conditions (Degraeve and Moutschen, 1984). The reduction in cell count/confluence in vitro for malathion was approximately 50%. Malathion is not carcinogenic in rodents (Ashby and Tennant, 1988). In summary, concentrations that are quite cytotoxic in vitro (e.g. ⩾ 50% cell killing, Fig. 7) are sometimes required to detect chemicals that are clastogenic in vivo.

A further difficulty in attempting to extrapolate from in vitro tests to the situation in vivo is that clastogenesis/cytotoxicity ratios in vitro may differ considerably between different cell types. For

TABLE 11

RELATIONSHIPS BETWEEN CHROMOSOME ABERRATION YIELDS AND CELL KILLING (LOSS OF CFE) IN HUMAN SKIN FIBROBLASTS TREATED IN VIVO WITH ANTI-TUMOUR AGENTS [a]

From Parkes and Scott (1982).

Chemical	Concentration [b] (μM)	Aberrations per 100 cells			CFE %
		Peak [c]	24 h [d]	Mean [e]	
Nitrogen mustard	1.0	10.2 (36 h)	0	4.6	20
Adriamycin	0.45	11.0 (6 h)	3.0	3.9	20
Daunorubicin	0.15	8.5 (6 h)	2.0	4.4	45
Daunorubicin	0.20	44.0 (6 h)	2.0	11.8	25
Daunorubicin	0.30	100.0 (6 h)	16.0	15.2	13

[a] Mitotic index data given in Fig. 5.
[b] Treatments were for 1 h.
[c] The time of maximum aberration frequency is given in brackets.
[d] Yield at 24 h given because this is a sampling time regularly used in clastogenicity tests.
[e] The mean yield between 6 and 48 h post-treatment.

example, Parkes and Scott (1982) examined in detail the relationship between chromosome aberration yields, CFE and MI in cultured human skin fibroblasts treated with anti-tumour agents which are clastogenic in human lymphocytes or bone-marrow cells in vivo (Table 11). Aberration yields were determined at 6 hourly intervals from 6 to 48 h after treatment. Although cell killing levels ranged from 55 to 87%, the average aberration yield over the entire sampling period did not exceed 15 aberrations per 100 cells. At 24 h, a time commonly used in testing, a direct comparison is possible between human fibroblasts (Table 10) and CHO cells (Table 11) in their response to adriamycin. The aberration yield at 20% survival in human fibroblasts was only 3% and the MI was reduced to about 40% of the control value (Fig. 5). In striking contrast, at the lowest survival level achieved in the assay in CHO cells, 32% CFE, the CA yield was 85 per 100 cells and there was actually an increase in MI relative to the control value. In human fibroblasts even at the peak frequency, at 6 h, the CA yield was only 11% at 20% survival. In another study in human fibroblasts exposed to MMC, only a 20% CA yield was observed at 80–90% cell killing and a 50% reduction in MI after a continuous 48-h exposure (Scott and Roberts, 1987). This level of chromosome damage was achieved with virtually no loss of viability in CHO cells (Table 10, Fig. 7) and with no reduction in MI (at least 24 h, Table 10). Clearly, if human fibroblasts are used in cytogenetic testing, then for these classes of chemicals highly cytotoxic concentrations must be used to detect clastogenic activity. In fact, human fibroblasts are rarely used, but human lymphocytes are, and it would be of value to examine clastogenesis/cytotoxicity ratios in vitro in this system for chemicals which are clastogenic in vivo.

One aim of the investigation of Armstrong, Galloway et al. was to examine clastogenesis/cytotoxicity relationships in vitro for chemicals whose rodent carcinogenicity is known (see key to Fig. 7). MMC is a rodent carcinogen (Ikegami et al., 1967) as is 2,4-DAT (cited by Ashby and Tennant, 1988). 2-Aminobiphenyl is a 'weak' carcinogen, classed as questionable by Lewis and Tatken (1979), and as negative in rats but positive in female mice and equivocal in male mice, by Hasemann et al. (cited in Ashby and Tennant, 1988). Eugenol is an equivocal carcinogen (rodent liver) and 8-HQ is a non-carcinogen (Tennant et al., 1987). As discussed above, for the rodent carcinogens MMC and 2,4-DAT, in vitro clastogenicity was detected with little or no cell killing, whereas for the weak carcinogen 2-ABP, the equivocal carcinogen eugenol and the non-carcinogen 8-HQ, concentrations that caused substantial acute killing or reductions in CFE were required to detect chromosome aberrations in vitro. At first glance, it might seem that the stronger carcinogens induced aberrations in vitro at less toxic doses than the weak or non-carcinogens. However, the non-carcinogen 2,6-DAT (Tennant et al., 1987) was only mildly cytotoxic at clastogenic doses in vitro. [Note that this was a case where concentrations above 10 mM were required in vitro to detect aberrations at the 24-h sampling time; the osmolality was not markedly increased. Gulati et al. (1989) were able to detect higher frequencies of aberrations with 2,6-DAT · HCl at 7.7 mM, at a 17-h sampling time in CHO cells.] It is clear, therefore, that clastogenic non-carcinogens can exert their effect without overwhelming toxicity, and cannot be distinguished

from clastogenic carcinogens on the basis of cytotoxicity alone.

There is need to examine, in detail, the relationships between the *type* of chromosome damage induced by chemicals in in vitro tests and the in vivo response. In preparing their review of in vitro clastogens, Ishidate et al. (1988) found that such information was often missing from published reports so they attempted such an analysis using only data for CHL cells from their own studies. In general they found that chemicals which were most efficient in inducing exchange-type aberrations were more likely to be rodent carcinogens than chemicals which induced predominantly gaps and breaks. [This may be because chromosomal deletions are more likely to be cell lethal than exchange events, since exchanges of the symmetrical type involve no loss of chromosomal fragments at mitosis and have now been clearly implicated in malignancy (Heim and Mitelman, 1987). Also, gaps may not reflect genomic damage relevant to carcinogenicity.] There was also a tendency for substances which were active only at high concentrations to induce more gaps and breaks than exchanges. However, there are exceptions; urea has an LEC value of 200 mM (12 mg/ml) in CHL cells but induces a high frequency of exchanges (Ishidate, 1988) as does sodium chloride in CHO cells at concentrations above 200 mM (Galloway et al., 1987a). Further evaluation of aberration type in relation to in vivo response is needed in different cell types and at different sampling times, with various types of clastogen (direct and indirect) before any general conclusions can be drawn.

From our consideration of the very limited quantitative data relating clastogenicity to cytotoxicity in vitro, it follows that, in our present state of knowledge, this relationship cannot be used to predict in vivo response. There is simply insufficient information to determine if there should be an upper limit of cytotoxicity for in vitro testing and, if so, what the upper limit should be. However, in the two examples discussed above, malathion and 8-HQ, for which positive results in CHO cells were obtained only at toxic levels and for which in vivo clastogenicity had been demonstrated, a reduction in cell growth of up to 50% (at the time of harvest for aberration analysis) was required to detect aberrations. For 8-HQ, CFE was reduced by 75% at clastogenic doses. In practice, the upper limit of testing in fibroblasts or lymphocytes is determined by the degree of reduction in the mitotic index at the chosen sampling time(s). With a typical MI of 5–10% a reduction of 75% is probably the maximum tolerable to give sufficient cells for analysis.

3.6. Cytotoxicity and mammalian cell mutation assays

For mutation assays, published guidelines generally recommend that the assay extend into the cytotoxic range. The OECD guidelines (1983) specify that mutation assays should extend to 'very low survival'. In the U.S. E.P.A. Gene-Tox Committee reports on mutation assays, upper limits of doses giving not less than 10% survival were recommended for mutations at the TK locus in L5178Y mouse lymphoma cells (Clive et al., 1983) and HPRT mutations in Chinese hamster ovary (CHO) cells (Hsie et al., 1981). For the HPRT mutation assay in Chinese hamster V79 cells (Bradley et al., 1981) a lower survival limit of 1–10% was suggested, but the authors pointed out the problems of trying to detect mutations in the small samples of cells remaining at survival levels below about 10%. Clearly, estimation of mutation frequency can become unreliable at very low survival (e.g. $< 10\%$), because of the small sample size and increasing variability. Also, pre-existing mutants may be selected because very slight differences in growth efficiency between mutant and wild type cells can have disproportionately large effects on final mutation frequency because by chance alone one can obtain an entirely spurious increase in mutations if a clone of mutant cells outgrows the rest of the depleted culture.

The reproducibility of results at high toxicity in the TK-locus mutation assay in mouse lymphoma cells has been discussed recently by Caspary et al. (1988). Several authors of papers on this system argue that results are unreliable with relative total growth (RTG) at less than 5% (Oberly et al., 1984), 10% (Clive et al., 1983) or 20% (Amacher et al., 1980). RTG is the product of the relative growth in suspension in the two-day expression period, and the subsequent cloning efficiency in soft agar which is done in parallel with the mutant selection (see Mitchell et al., 1988). Caspary et al.

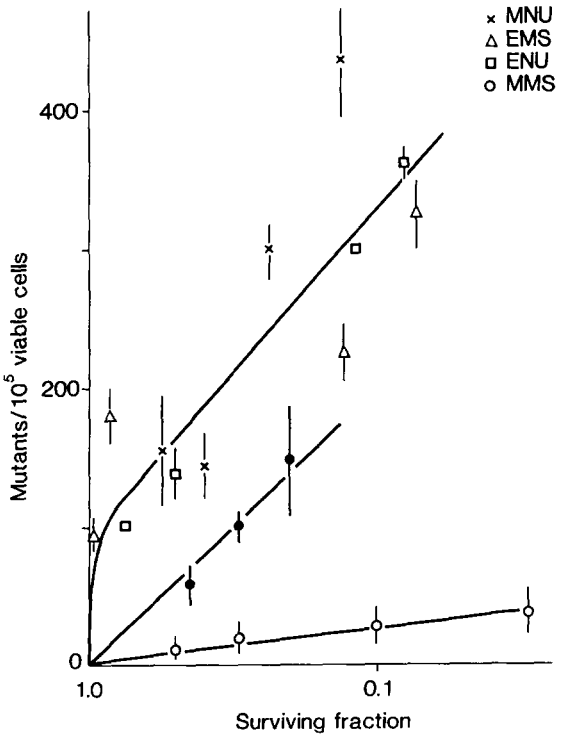

Fig. 8. Relationship between induced frequency at the HPRT locus and cell survival in V79 Chinese hamster cells. (Fox, 1980)

(1988a) analysed the variability in their data on 800 experiments with L5178Y cells, and examined the effect of toxicity on variability between replicate cultures, by comparing the coefficients of variation at all levels of toxicity using the three measures available; growth at one day and at two days, and cloning efficiency (CE). They concluded that under their protocol it was statistically valid to use results of mutation assays with RTG as low as 1%, provided that the CE was not less than 10% and that the observed increase in mutants per survivor was supported by an absolute increase in the number of mutants.

Mutagenicity/cytotoxicity ratios differ greatly for different mutagens (e.g. Carver et al., 1979, 1983; also Fig. 8). For example, methyl methanesulphonate (MMS) is known as a 'toxic mutagen' and ethylnitrosourea (ENU) as a potent mutagen with low cytotoxicity. A database is lacking that would allow comparison of in vitro and in vivo mutation data, since in vivo measurement of somatic mutations, e.g. at the HPRT locus, has been applied to very few compounds. For in vivo mutation assays, mutagens that have proven most effective are specifically chosen for lack of toxicity, e.g. ENU, which is a potent mouse germ cell mutagen (Russell et al., 1979) and the only chemical mutagen for which in vivo mouse lymphocyte HPRT mutation data are available (Burkhart-Schultz et al., 1989). Some assessments have been made, however, of in vitro mutagenic potency (in terms of cytotoxicity) compared with carcinogenicity in rodents, for the thymidine kinase locus (TK mutations) in L5178Y cells which detects both point mutations and clastogenic events (Applegate and Hozier, 1987).

Data were examined from a study of a large series of compounds tested under the U.S. National Toxicology Program many of which are described by Mitchell et al. (1988) and Myhr and Caspary (1988) (Figs. 9 and 10, data kindly provided by W. Caspary, N.I.E.H.S.). Fig. 9 shows data for positive mutation assays; the RTG at the lowest dose that gave a detectable mutation response is plotted, and it is clear that there is a whole spectrum, from compounds that are detectable as mutagenic only at low survival, to those that are mutagenic at non-toxic levels. For the majority of compounds however, there is a degree

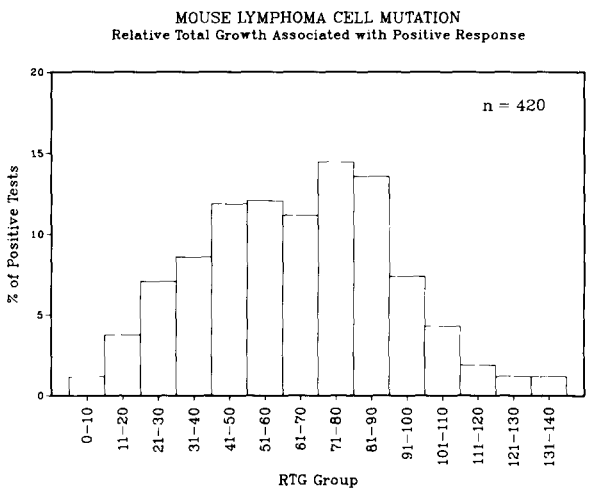

Fig. 9. Level of cytotoxicity (relative total growth, RTG) at which mutations at the TK locus in mouse lymphoma cells were detectable. Data are for 420 chemicals (kindly provided by W. Caspary, NIEHS).

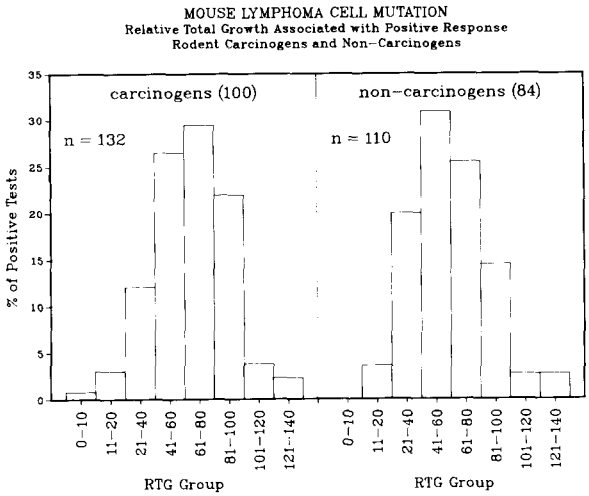

Fig. 10. As for Fig. 9 but chemicals are divided into rodent carcinogens and non-carcinogens.

of cytotoxicity associated with the concentrations of chemicals required to produce a detectable mutagenic response. In Fig. 10, the data for positive mutation assays have been broken down into rodent carcinogens and non-carcinogens, and there is no obvious difference between the patterns of association of toxicity with mutagenicity. In particular, some carcinogens were detectable only at RTG of < 20%. Similarly, Wangenheim and Bolcsfoldi (1988) tested 50 compounds in the mouse lymphoma (L5178Y) cell mutation assay, and examined their own data and published data for 105 compounds for the relationships between mutagenicity, toxicity and carcinogenicity. Of 33 compounds that were positive at less than 20 mM, 8 were positive only at quite high toxicity, i.e. when the RTG was in the 10–20% range. Two of these 8 were carcinogens, and the other 6 were weak, non-carcinogenic, or untested. Similarly, in the literature that they reviewed, 20 of the 105 compounds had 2–4-fold increases in mutation frequencies in the 10–20% RTG range, and of these 20, 8 were known carcinogens.

The data of Wangenheim and Bolcsfoldi and those of the NTP (Mitchell et al., 1988; Myhr and Caspary, 1988) therefore agree on the observation that some rodent carcinogens are detected as in vitro mutagens in the L5178Y TK-locus assay only when the RTG is less than 20%.

In the work of Mitchell et al. (1988) and Myhr and Caspary (1988) there were some positive results at very low survival, but also some very toxic non-mutagens. These included lithocholic acid with S9 activation, 4,4′-methylene-bis-2-chloroaniline without S9 and *p*-rosaniline HCl with S9 (Myhr and Caspary, 1988). This demonstrates that mutation is not an inevitable consequence of cytotoxicity. These toxic-but-negative tests were in the minority; of 29 negative assays without S9 and 12 with S9, only 2 and 3, respectively, were classed as negative and toxic.

In another in vitro mutation system, HPRT mutations on V79 Chinese hamster cells, some carcinogens (e.g. MMS, Fig. 8) are detectable only at highly cytotoxic concentrations, the latter measured as loss of CFE (reviewed by Fox, 1980). In this review Fox also draws attention to the different quantitative relationships between induced mutation frequency and cell killing in different cell types treated with the same chemical, and between different genetic loci.

In conclusion, the upper limits for cytotoxicity for in vitro mutation assays must be set to take account of the variability at small sample sizes, and the data examined to see if there is a real increase in the number of mutant colonies. The data discussed suggest, however, that mutation assays can and should be carried out at quite low survival. Although mutations can be induced by indirect mechanisms (e.g. cycloheximide, see Table 9 and footnote) evidence is lacking that these occur *only* above a certain level of cytotoxicity.

3.7. Cytotoxicity and DNA-strand breaks

DNA-strand breakage is sometimes used as a measure of genotoxicity (e.g. Williams et al., 1985) because, although double-strand breaks may be cell lethal (Leenhouts and Chadwick, 1984; Bryant, 1985; Radford, 1986) they are also believed to be the precursors of chromosome aberrations (Natarajan et al., 1980; Bryant, 1984).

Sina et al. (1983) have addressed the question of whether the quantitative relationships between DNA-strand breakage and cytotoxicity in vitro can be used to predict the carcinogenicity of chemicals. They measured DNA single-strand breaks (ssb's) in freshly isolated rat hepatocytes at the end of a 3 h treatment and at the same time

measured cytotoxicity by loss of membrane integrity (Section 3.3a). They found that all of 51 'relatively strong (rodent) carcinogens' induced ssb at minimally cytotoxic doses (i.e. less than 30% cytotoxicity) and were designated Class I compounds. Of 16 chemicals which caused ssb's concomitant with a significant reduction in viability (> 30%, designated Class III chemicals) 12 were weak carcinogens (i.e. tumourigenicity was dependent upon specific conditions such as species, strain, sex, high doses, route of administration, etc.) and 4 were non-carcinogens. In later studies these investigators found that DNA double-strand breaks (dsb's) induced by Class I and Class III compounds were only detectable at doses inducing considerable cytotoxicity (Bradley et al., 1987). They argued that the breaks detected as ssb in the alkaline elution assay of Class III compounds were actually the dsb detected at highly toxic doses in the subsequent assay by neutral elution. They suggest that these dsb's arise as a consequence of 'toxic cellular damage' which results in disruption of lysosomes, releasing DNA nucleases. Possibly, relatively non-specific 'damage' such as ionic imbalance, energy depletion or protein denaturation (Section 3.2) could lead to lysosomal leakiness. The unscheduled release of lysosomal enzymes other than DNA nucleases would be expected to contribute to the observed cytotoxicity.

On the scheme presented in Fig. 4, the Class I chemicals of Bradley and colleagues would produce 'DNA changes' (in this case strand breakage) predominantly by 'direct' mechanisms of DNA interaction, inducing ssb's at relatively non-toxic doses. However, a minor 'indirect' pathway for strand breakage (dashed line with arrow), detectable at high doses, would be *via* 'non-DNA changes' leading both to dsb's and cytotoxicity. This latter pathway would be the mechanism whereby Class III chemicals are effective i.e. on this scheme they would be classified as 'indirect genotoxins' and, the authors argue, might show a threshold in some cases. Bradley (1985) has suggested that this indirect genotoxicity may be important in rodent carcinogenicity assays at high doses, where limited double-strand DNA breakage in sublethally damaged cells could be a mechanism for oncogenic transformation in vivo.

Some support for the proposed role of lysosomal nucleases in indirect DNA breakage has come from the demonstration that the lysosomotrophic detergent *N*-dodecylimidazole can induce dsb's and chromosomal aberrations (Bradley et al., 1987). Certainly bacterial restriction endonucleases can induce chromosomal aberrations in mammalian cells (e.g., Natarajan and Obe, 1984; Winegar and Preston, 1988), and DNAase I, when supplied to cells enclosed in liposomes to allow active enzyme to reach the nucleus, can induce cytotoxicity, morphological transformation, mutations and chromosome aberrations (Zajek-Kaye and Ts'o, 1984; Nuzzo et al., 1987). Under conditions of 'unbalanced growth', where protein synthesis continues but DNA synthesis is inhibited by exposure to hydroxyurea or excess thymidine, Sawecka et al. (1986) reported an increase in enzyme activity of DNAase II both in cells and in the medium, and postulated that the nuclease activity was responsible for the associated DNA breakage. Ayusawa et al. (1988) also presented evidence that the DNA breaks in thymidylate-starved cells result from endonuclease activity.

The general applicability of the nuclease theory to other cytotoxic compounds is not yet known. It is also unknown whether *limited* endonuclease damage is possible, such that some damaged cells are able to survive — a crucial point, since there are no mutagenic or carcinogenic consequences if the cells die or cannot reproduce.

3.8. In vivo cytotoxicity, indirect genotoxicity and carcinogenesis

There is evidence that cytotoxicity in vivo may itself play a role in carcinogenesis (Swenberg and Short, 1987; Zeise et al., 1985, 1986), perhaps by inducing chronic cell proliferation or inflammation. We have asked the question whether potentially toxicity-mediated tumours are associated with 'non-genotoxic' or genotoxic chemicals, and if genotoxic in vitro, is there any evidence that they might be directly genotoxic? It has been postulated that in rodent carcinogenicity assays at high doses limited double-strand breakage in sublethally damaged cells, e.g., as a result of lysosomal leakiness, could be a mechanism for oncogenic transformation (Bradley, 1985). The possible

TABLE 12

GENOTOXICITY DATA IN VITRO FOR CARCINOGENS INDUCING TARGET-ORGAN TOXICITY

'Toxicity associated carcinogen' [a]	Genotoxicity in vitro [b]			
	Ames	Mut	CA	SCE
1,4-dichlorobenzene	neg [c]	ND	ND	ND
ethyl acrylate	neg	pos	pos	pos
isophorone	neg	pos	neg	pos
melamine	neg	neg	neg	equivocal
pentachloroethane	neg	pos	neg	pos
1,1,1,2-tetrachloroethane	neg	pos	neg	pos
2,6-xylidine	ND	ND	pos [d]	ND
allyl isothiocyanate	equiv.	pos	pos	pos
11-aminoundecanoic acid	neg	neg	neg	pos
chlorodibromomethane	neg	pos	neg	pos
3-chloro-2-methylpropene	neg	pos	pos	pos
C.I. Disperse Yellow 3	pos	pos	neg	pos
C.I. Solvent Yellow 14	pos	pos	neg	pos
D and C Red 9	pos	neg	neg	neg
diglycidyl resorcinol ether	pos	pos	pos	pos
dimethyl hydrogen phosphite	pos	pos	pos	pos
monuron	neg	equiv.	pos	pos
pentachloroethane	neg	pos	neg	pos
polybrominated biphenyl mixture	neg	neg	neg	neg
propylene oxide	pos	pos	pos	pos

[a] Hoel et al. (1988); Chemicals inducing either hyperplasia or toxic target-organ lesions for all species and both sexes at dose levels showing increased tumour incidence. The chemicals above the line were those with toxic lesions in all target organs showing chemically induced neoplasia, and likely to have been responsible for the types of tumours observed.

[b] Tennant et al. (1987); Ames, Salmonella test; Mut, mutation (TK) in L5178Y cells; CA, chromosome aberrations in CHO cells; SCE, sister-chromatid exchanges in CHO cells.

[c] Ashby et al. (1989).

[d] Galloway et al. (1987).

ND, no data.

association between chronic toxicity and tumour induction for 53 rodent carcinogens has been analyzed by Hoel et al. (1988). Of the 53 carcinogens considered, they identified 7 compounds which exhibited the types of target organ toxicity that might have been involved in induction of the observed tumours (Table 12). All 7 were negative in the Ames test. Of the 6 for which in vitro chromosomal aberration data are available, 2 were positive. Hoel et al. (1988) also listed 15 compounds that caused tumours and also caused preneoplastic responses such as hyperplasia apparently associated with chronic toxicity. The 13 for which genotoxicity data are available are listed in Table 12. Of these, about half were positive in the aberration test in vitro. Clearly these 'toxic carcinogens' are not necessarily 'non-genotoxic' in vitro; insufficient data exist to determine whether any of these might have induced genotoxicity indirectly in vitro, and whether toxicity might have been responsible for both the genotoxicity and carcinogenicity of any of these chemicals. It will be interesting to obtain in vivo clastogenicity data on these.

3.9. Conclusions and recommendations

Since human exposure to environmental genotoxins is unlikely to be at concentrations which induce much cytotoxicity, the relevance of in vitro genotoxicity at cytotoxic doses can be questioned. However, in using the rodent models for human genotoxicity it is clear that some rodent genotoxins can only be detected in in vitro tests at relatively high levels of cytotoxicity (Section 3.5).

Genotoxicity/cytotoxicity ratios in vitro cannot discriminate between chemicals that are positive or negative for genotoxicity or carcinogenicity in vivo. This is not surprising in view of the severe limitations of the quantitative data relating genotoxicity to cytotoxicity in vitro. We have seen that most measurements of cytotoxicity and some genotoxic endpoints are markedly dependent upon sampling time, that genotoxicity/cytotoxicity ratios may differ very considerably between cell types even for the same chemical (Tables 10 and 11) and between different gene loci in mutation assays (Section 3.6).

Nevertheless, if a chemical is found to be genotoxic without much cytotoxicity in an in vitro test it should be regarded as potentially active in vivo. On the other hand, to designate a chemical as being unlikely to have in vivo activity by virtue of associated cytotoxicity would require extensive investigations of genotoxicity/cytotoxicity relationships in a large number of in vitro tests. To further designate a chemical as being without human risk it would be necessary to establish that there was a true threshold in the relationship between genotoxicity and cytotoxicity at a level of cytotoxicity which could not be achieved in man. In vivo testing for genotoxicity would be important in assessing the results. If thorough in vivo testing gave a negative result, and genotoxicity in vitro was detectable only at highly cytotoxic concentrations (perhaps in only one assay and not in others) it would not be unreasonable to conclude that human risk would be very low.

In considering the relationship between genotoxicity and concentration (Section 2) we concluded that, at high concentrations of non-DNA-reactive chemicals, artefactual genotoxicity could arise because of osmolality changes in the culture medium and that such effects do not occur at lower concentrations i.e. there is a true threshold response. We also concluded that such effects are unlikely to be relevant to human exposure or human risk. We must ask whether similar artefactual responses occur at high levels of cytotoxicity but not at lower levels i.e. if there are circumstances in which there is a true threshold in the relationship between genotoxicity and cytotoxicity. If cell death is taken as the cytotoxic endpoint it is difficult to envisage mechanisms leading to cell death which are accompanied by genotoxicity *only* at high levels of killing and not at lower levels. For direct genotoxins (Fig. 4) inducing, for example, CA or gene mutations, this would require that at lower concentrations the induced DNA lesions produce lethal but no clastogenic or mutagenic events and that the latter two are only produced when the burden of DNA lesions is above a certain level. If DNA strand breakage is the genotoxic endpoint it would require that cell death at lower concentrations be caused by mechanisms not involving DNA damage. For indirect genotoxins (Fig. 4) the non-DNA changes at lower concentrations would have to lead exclusively to cell death and, only at higher doses, to both cell death and DNA changes. A theoretical possibility for the latter is with chemicals for which there is a threshold in the genotoxicity/concentration relationship e.g. enzyme inhibitors or chemicals which produce active oxygen species or cause leakiness of lysosomes (Section 2). If, in addition to this mechanism such a chemical had a second independent mechanism of cytotoxicity, unaccompanied by genotoxicity, at lower concentrations then a true threshold in the genotoxicity/cytotoxicity relationship would be seen and could possibly be used to assess the likelihood of human risk. However, no chemical with these properties has yet been identified.

It follows that in our current state of knowledge we are unable to define upper limits of cytotoxicity for in vitro testing other than on purely practical grounds (e.g. having sufficient cells for analysis in clastogenicity testing, statistical accuracy in mutational assays) but we are not aware of any artefactual mutagenicity or clastogenicity occurring only above a threshold level of cytotoxicity, though there may be examples for DNA double-strand breakage. Further quantitative analyses of genotoxicity/cytotoxicity relationships are urgently required.

4. Genotoxicity of liver microsome activation systems (Table 13)

Most chemical mutagens are biologically inert unless metabolically activated. In in vitro tests, the capacity for metabolic activation is normally provided in the form of 'S9 mix'. S9 is the super-

natant after centrifugation of a liver homogenate (usually rodent) at 9000 *g* and comprises microsomes, which carry the enzymes required for metabolic activation and a cytosolic fraction, which can be removed after further centrifugation to isolate the microsomes. A source of NADPH is also required and this is generated in the S9 mix by cofactors, i.e. NADP and either glucose 6-phosphate or isocitrate as the energy source. It is becoming clear that under certain circumstances metabolic activation systems can themselves be genotoxic. It is important to determine the conditions under which mutagens in the activating systems can arise to avoid obtaining spurious positive results for chemicals undergoing genotoxicity testing.

4.1. Bacterial systems

Reports indicating that S9 preparations are mutagenic or cytotoxic in bacterial systems are rare. It has, however, been commonly observed that higher spontaneous reversion frequencies occur in the presence than in the absence of S9 (e.g. Ames et al., 1975). In the Salmonella/microsome assay this has been interpreted as a 'feeding effect' *via* the presence of histidine in the S9 preparation (Venitt et al., 1984). The phenomenon was considered, however, in some detail by Peak et al. (1982) who concluded that S9 either contained a mutagen or activated some component of the plating medium to a mutagen. The latter explanation was supported by the work of Dolora (1982) and of Maron et al. (1981) who both attributed the effect to the presence of indirect acting mutagens (i.e. agents metabolically activated to mutagens) carried over in the nutrient broth. This explanation, however, could not account for the results of Rossman and Molina (1983) who consistently found a small, but statistically significant increase in background reversion of *E. coli* WP2 induced by the presence of S9. The effect was not seen using *S. typhimurium* strain TA100. The result was shown not to be due to a feeding effect or the presence of indirect acting mutagens in nutrient broth. The mutagenic activity could be removed by dialysis of the S9 and it therefore appeared that the S9 itself contained a mutagen.

Although the toxic effects of S9 preparations in some mammalian systems are well documented, such effects are not commonly associated with bacteria. This may be partly due to the fact that toxicity can only be detected in plate incorporation tests if it is pronounced. It is noteworthy, however, that S9 has been shown to be cytotoxic to Ames Salmonella strains in liquid suspension assays where cell survival is quantitatively and sensitively assessed (Rosenkranz et al., 1980).

That bacterial mutagens can be generated by isolated liver microsome preparations (and therefore, potentially by S9) was shown by Akasaka and Yonei (1985) who incubated *E. coli* WP2 uvrA (pKM101) cells in a preparation of microsomes from rat liver containing NADPH and Fe^{2+}. Mutation was measured following incubation periods of up to 60 min. Under these conditions the microsomes were both strongly cytotoxic and mutagenic. The mutagenicity was attributed to lipid peroxidation (Section 4.3) although the genotoxic product(s) of lipid peroxidation in this system was (were) not identified.

4.2. Mammalian systems

The cytotoxicity of S9 mix to human peripheral blood lymphocytes is well documented (Bimboes and Greim, 1976; White and Hesketh, 1980; Madle and Obe, 1977; Madle, 1981) although, again, the nature of the cytotoxic component has not been considered in depth. Tan et al. (1982) using Chinese hamster ovary (CHO) cells, compared the effects of two activation systems where the activating enzymes were supplied as either S9 or as purified microsomes. Whereas the former was not cytotoxic over a 5-h incubation period, the preparation utilising isolated microsomes reduced cell survival by approximately 60%. These results are comparable with those of Akasaka and Yonei (1985, cited above) using *E. coli*. Cytotoxicity was again attributed to the products of lipid peroxidation but it is noteworthy that in contrast to *E. coli* the microsome preparations did not induce mutation (at the HPRT locus) in the CHO cells.

Genotoxic effects associated with metabolic activation systems are summarised in Table 13. These isolated reports must, of course, be considered against a large volume of data indicating that, under most circumstances, S9 does not have a marked effect on spontaneous point mutation or chromosome aberration frequencies, although

slightly higher background frequencies of both these end-points are commonly observed in the presence of S9 mix (Caspary et al., 1988b; Margolin et al., 1986).

The first clear recognition of the mutagenic potential of S9 in mammalian cells was made by Myhr and Mayo (1987) in the mouse lymphoma L5178Y mutation assay. Although S9 alone or S9 mix induced no measurable increase in mutation following a 'normal' 4-h treatment period, striking increases were observed when the exposure time was extended to 8 or 24 h. Similar increases occurred when cells were exposed for 4 h to S9 preparations which had been preincubated with growth medium for 18 h prior to treatment, indicating that a process was occurring within the S9 itself to produce a mutagenic substance. McGregor et al. (1988) were able to show that background mutation frequencies in L5178Y cells could also increase if the proportion of S9 in the S9 mix was increased. S9 concentrations up to 10 mg whole liver equivalents/ml were not significantly toxic or mutagenic but 12.5 mg whole liver equivalents/ml increased the mutant fraction and reduced survival (concentrations of S9 up to 25 mg/ml are commonly used in this assay).

Myhr and Mayo (1987) observed that S9-induced L5178Y mutants were predominantly 'small colony' type. These are thought to arise as a result of chromosomal aberrations. This is significant in view of the findings of Kirkland et al. (1989) that certain batches of Aroclor 1254-induced S9 produced chromosomal aberrations in their clone of CHO cells. The effect appeared to be dependent on the presence of NADP and G-6-P and was observed following a short (2 h) incubation period. The clastogenic activity of the S9 mix could be reduced by co-incubation with catalase or vitamin E implying that active oxygen species were involved. Oxygen radicals are an initial product of lipid peroxidation (Vaca et al., 1988). It was assumed that microsomal lipid peroxidation was the cause of the clastogenicity of the S9 mix.

4.3. Lipid peroxidation

Metabolic activation systems utilizing isolated microsomes appear to be much more likely to generate toxic and mutagenic species than S9 mix.

TABLE 13

GENOTOXIC EFFECTS IN VITRO ASSOCIATED WITH METABOLIC ACTIVATION SYSTEMS

End point	Cell type	Activation system	Treatment duration	Comment	Reference
Point mutation	*E. coli* WP2	S9 mix	(Plate incorporation	Increased mutation to Trp^+. Effect not seen in *S. typhimurium* TA100	Rossman and Molina (1983)
Point mutation	*E. coli* WP2 uvrA	Isolated microsomes NADPH + Fe^{2+}	up to 60 min	Increased mutation to streptomycin resistance	Akasaka and Yonei (1985)
Point mutation (HPRT locus)	CHO	Isolated microsomes + co-factors	5 h	Cytotoxicity but no mutagenicity observed	Tan et al. (1982)
Point mutation and/or CA	Mouse lymphoma	S9 mix	4–24 h	Mutant frequency increased after 8 h exposure	Myhr and Mayo (1987)
Point mutation and/or CA	Mouse lymphoma	S9 mix	4 h	Mutant frequency increased with increasing S9 fraction in mix	McGregor et al. (1988)
Chromosome aberrations	CHO	S9 mix	2 h	Marked increases in chromosome aberrations observed	Kirkland et al. (1989)

CA, chromosome aberrations.

This may be due to the presence of an inhibitor of microsomal lipid peroxidation found in the cytosolic fraction of liver (Kamataki et al., 1974; Kotake et al., 1975; Player and Horton, 1978; Talcott et al., 1980). That microsomal lipid peroxidation can occur in S9 mix formulations, characteristic of those used in short-term testing, has, however, been clearly shown. Vaca and Harms-Ringdahl (1986) demonstrated that the rate of lipid peroxidation was higher in S9 mix prepared from rats fed diets rich in polyunsaturated fatty acids, could be stimulated by the presence of Fe^{2+} ions and was higher in S9 mix from Aroclor 1254-induced rats than uninduced animals. In contrast Paolini et al. (1983) found that lipid peroxidation occurred more readily in S9 mix from uninduced animals than in S9 mix from rats induced with β-naphthoflavone and phenobarbitone. It appears that marked changes in membrane lipid composition occur following induction, and this depends on the inducing agent. This may provide some explanation for the results of Kirkland et al. (1989) which indicated that batches of S9 mix from rats induced with Aroclor 1254 were clastogenic whereas S9 mix from rats induced with β-naphthoflavone and phenobarbitone was not.

The potential of S9 preparations to undergo lipid peroxidation must vary considerably among the different S9 mix formulations and treatment conditions used by different laboratories in in vitro tests. Media components (e.g. Fe^{2+} content) and concentration of cofactors are known to be important (Vaca and Harms-Ringdahl, 1986). Factors that accelerate microsomal lipid peroxidation have also been recognised and these include radiation, hyperoxia, nitrogen oxides and radical initiators (Buege and Aust, 1978).

4.4. Recommendations

Possible means of reducing or eliminating the genotoxic effects of S9 preparations include the addition of agents such as catalase or vitamin E to the incubation medium (Kirkland et al., 1989). BHA and EDTA are likely to be effective also because they can inhibit lipid peroxidation (Tan et al., 1982; Vaca and Harms-Ringdahl, 1986; Paolini et al., 1988). Simply reducing the duration of treatment may not be appropriate however, because long incubations (several hours) in the presence of S9 are required to detect some promutagens in mammalian cells (Sbrana et al., 1984; Machanoff et al., 1981).

Sensitivity to the products of lipid peroxidation must vary between mutagenic endpoints and this could explain why S9 induced mutagenesis is not more commonly observed. For example, active oxygen species are not mutagenic to the most frequently used *S. typhimurium* tester strains (TA1535, TA1538, TA1537, TA98, TA100) (Levin et al., 1982). Kirkland et al. (1989) found that the batches of S9 which caused elevated frequencies of chromosome aberrations in CHO cells gave normal mutation frequencies in Ames tests and mammalian cell mutation tests and normal levels of repair in unscheduled DNA synthesis assays. These same batches showed no evidence of clastogenicity with lymphocytes in whole blood culture. This may well have been attributable to culture conditions rather than cell type because active oxygen species have been shown only to be genotoxic in human lymphocytes when cultured following isolation from whole blood (Mehnert et al., 1984). At present, however, it is not possible to recommend the use of insensitive cell types or protective culture conditions because the peroxidation of membrane lipids is recognised as an indirect mechanism of genotoxicity. Phorbolmyristate acetate (Cerutti, 1985) and chromium chloride (Friedman et al., 1987) are examples of compounds which are thought to act in this way (Table 9). It seems unlikely that chemicals exist which initiate lipid peroxidation in liver microsomal membranes but not in other cell membranes and it is important that such 'membrane-active' agents are detected. Firstly, it must be clearly established that the genotoxicity of liver-microsome activation systems that has been observed can be attributed to lipid peroxidation. The next step should then be to develop methods for S9 induction and S9 mix formulations which make this less likely to occur.

5. Genotoxicity induced by extremes of pH (Table 14)

Non-physiological pH can not only influence the mutagenicity of many compounds (Zetterberg

et al., 1977; Whong et al., 1985; LeBoeuf et al., 1989) but can be mutagenic per se.

5.1. Non-mammalian systems

Extremes of pH (3–10) have been shown not to induce mutation in the Ames test in the presence or absence of S9 using standard plate incorporation or pre-incubation methods (Tomlinson, 1980; Cipollaro et al., 1986). Prolonged incubation (at least 4 h) prior to plating under strongly alkaline conditions (pH 10) does however result in mutagenesis in certain bacterial strains (Musarrat and Ahmad, 1988). The effect was observed in Salmonella strains TA97, TA102, TA104 and *E. coli* K12 but not in TA98 or TA100. From the strain specificity it was inferred that alkali treatment caused damage preferentially at A–T-rich regions in the DNA and, from liquid holding experiments following exposure, that this damage could be repaired.

As yet, no evidence has been obtained for pH-related increases in point mutation in yeast (Tomlinson, 1980; Nanni et al., 1984; Whong et al., 1985) though effects have not been measured outside the range 3–9. Low pH has, however, been shown to induce gene conversion in *Saccharomyces cerevisiae* (Nanni et al., 1984) and high pH to increase point mutation in the fungus *Cladosporium cucumerinum* (but not in *Aspergillus nidulans*, Nirenberg and Speakman, 1981).

Sea urchin embryos appear to be very sensitive to the genotoxic effects of low pH (Cipollaro et al., 1986). A short exposure to pH 6.0 was sufficient to induce a variety of mitotic abnormalities (anaphase bridges, lagging chromosomes, multiple breaks and multipolar mitoses).

The clastogenic effects of low pH are now well documented in *Vicia faba* root tips although exposure to pH 4 is required before significant increases in aberrations are observed (Bradley et al., 1968; Zura and Grant, 1981).

5.2. Mammalian systems

In mammalian systems the genotoxic effects of pH appear to be strongly enhanced by the presence of S9. Low pH has been shown to induce chromosome aberrations in CHO cells after treatment with hydrochloric or acetic acid (Thilager et al., 1984). No increase in aberrations was observed in the absence of S9 at pH 5.5 but, in its presence, large numbers of aberrations were induced. These data were interpreted to indicate that S9 could be broken down into clastogenic products. It is now clear, however, that the presence of S9 is not a prerequisite for clastogenesis. Thus Morita et al. (1989a) showed that at low pH (5.5 or less), aberrations were induced in CHO cells in both the absence and presence of S9 though the effect was enhanced by S9. Phosphate-buffered saline acidified to pH 5.2 was also clastogenic indicating that the effect was not due to decomposition products of the culture medium. In addition, although no clastogenic activity was observed over the pH range 7.3–10.9 without S9, aberrations were observed at pH 10.4 with metabolic activation. Further studies (Morita et al., 1989b) indicated that reducing the pH of the medium and then neutralising to pH 6.4 or 7.2 or using an organic buffering system removed the clastogenic effect.

Chromosomal effects are also induced in other cell types. Shimada and Ingalls (1975) exposed human lymphocytes for 3 h to pH levels over a range 6.5–8.8. A statistically significant increase in hyperdiploid cells was observed in cultures exposed to pH 6.5–6.9 providing evidence for pH-induced non-disjunction. Endoreduplication was also significantly increased at low pH (< 7) but not at high pH. The authors claimed that chromosome structural damage (including exchange aberrations) was observed at both low and high pH levels but this was not quantified. In the light of these results the results of Sinha et al. (1989) are unexpected. These authors found that exposure of rat lymphocytes to pH levels as low as 2.73 or as high as 9.97 for 4 h in the absence or presence of S9 did not result in clastogenesis. Mitotic inhibition (which, interestingly, was more marked in the presence of S9) was evident at extreme pH levels indicating that it would not have been possible to expose the cells to more severe conditions of pH. It is not clear why rat and human lymphocytes should respond differently because culture conditions and sampling times were not dissimilar in the two experiments (both were cultured as whole blood).

Clastogenicity was used to explain increases in spontaneous mutation frequency at the TK locus

TABLE 14

GENOTOXIC EFFECTS IN VITRO ASSOCIATED WITH EXTREMES OF pH

End point	Cell type	Chemical	S9	pH range studied	Treatment duration (h)	Effective pH	Comment	Reference
Point mutation	*Salmonella typhimurium* TA100, TA1535	$H_3PO_4/KH_2PO_4/K_2HPO_4$	–	3–8	1–4	None	Pre-incubation	Tomlinson (1980)
Point mutation	*Salmonella typhimurium* TA98, TA97, TA100, TA120, TA1535	H_3PO_4/H_2SO_4	–/+	4.0–9.0	1 (pre-incubation)	None	Pre-incubation or plate incorporation. Toxicity at 5.0 and less	Cipollaro et al. (1986)
Point mutation	*E. coli* K12, *Salmonella typhimurium* TA104, TA102 TA97	Tris/NaOH	–	8–10	6	10	Pre-incubation Little or no effect with TA98 or TA100	Musarrat and Ahmad (1988)
Point mutation	*Aspergillus nidulans*	Lactic acid/KOH	–	5.0–6.8	8	None		Nirenberg and Speakman (1981)
Point mutation	*Cladosporium cucumerinum*	Lactic acid/KOH	–	5.0–6.8	20	6.8	'physiological' pH is 5.0–5.2	
Point mutation	*Neurospora crassa*	$H_3PO_4/KH_2PO_4/K_2HPO_4$	–	3–8	2–4	None		Tomlinson (1980)
Gene conversion	*Saccharomyces cerevisiae*	HCl/NaOH	–	5–8	40	5.8		Nanni et al. (1984)
Point mutation	*Saccharomyces cerevisiae*	HCl/NaOH	–	5–8	2	None		Nanni et al. (1984)
Point mutation	*Neurospora crassa*	Na_2HPO_4/KH_2PO_4	–	5–9	3	None		Whong et al. (1985)
Mitotic abnormalities	Sea urchin embryos	HCl, H_3PO_4, H_2SO_4	–	5.5–8.5	1 (or more)	6.0 (approx)		Cipollaro et al. (1986)

Chromosome aberrations	*Vicia faba*	Sucrose/KOH/HCl	–	3.0–7.5	4	4		Bradley et al. (1968)
Chromosome aberrations	*Vicia faba*	Citric–citrate buffer	–	4–7	–	4	Continuous exposure	Zura and Grant (1981)
Aneuploidy	Human [a] lymphocytes	Lactic acid/NaOH	–	6.5–8.8	3	< 7.0 and > 8.4		Shimada and Ingalls (1975)
Endoreduplication	Human [a] lymphocytes	Lactic acid/NaOH	–	6.5–8.8	3	< 7.0		Shimada and Ingalls (1975)
Chromosome aberrations	Rat lymphocytes [a]	NaOH/HCl	–	5.10–9.97	4	None	Toxicity, but no clastogenicity	Sinha et al. (1989)
			+	4.17–10.01	4	None	Toxicity, but no clastogenicity	
Chromosome aberrations	CHO	Acetic acid	+	5.28–7.24	2	5.5	No effect minus S9	Thilager et al. (1984)
		HCl	+	5.00–5.75	2	5.25	No effect minus S9	
Chromosome aberrations	CHO	KOH	+	7.3–10.9	6	10.4	No effect minus S9	Morita et al. (1989a)
		NaOH	+	7.4–10.8	6	10.8	No effect minus S9	
		HCl	–	5.3–7.4	24	5.5		
			+	5.5–7.4	6	6.0		
		H_2SO_4	–	3.5–7.4	24	4.5		
			+	4.5–7.4	6	6.2		
		NaOH	–	6.0–7.0	24	6.2		
		HCl	–	5.8–6.6	24	6.2		
Chromosome aberrations	CHO	Formic acid, lactic acid, acetic acid	–	5.9–7.1	24	6.0 (approx.)		Morita et al. (1989b)
			+	5.9–7.1	6			
Point mutation and/or CA	Mouse lymphoma	Good's buffers	+	6.4–7.6	4	6.8	No effect minus S9	Cifone et al. (1987)
		Good's buffers/HCl	–	6.3–7.5	4	6.3	2-fold increase	
			+	6.3–7.5	4	6.5	2.6-fold increase	
		Good's buffers/NaOH	+	7.3–8.8	4	8.3	2-fold increase (no effect minus S9)	

[a] In whole blood culture.
CA, chromosome aberrations.

in mouse lymphoma L5178Y cells following exposure to low pH (Cifone et al., 1984, 1985, 1987). The induced colonies observed were typically of the 'small colony' phenotype which implied that they arose as a result of chromosomal aberrations. This was confirmed by chromosome aberration analysis of low pH-treated cultures. The effect was greatly enhanced by the presence of S9 mix.

5.3. Mechanisms

The mechanism by which non-physiological pH levels cause genotoxicity is not clear but it has been known for many years that pH can influence the level of depurination of bacterial or viral DNA (Strack et al., 1964). Brusick (1986) noted that the fidelity of the DNA replication and repair enzymes may be reduced by extremes of pH and this could produce genotoxic effects. The data of Thilager et al. (1984) and Cifone et al. (1987) indicate that biologically active species may be produced under certain conditions in S9 mix at low pH.

5.4. Recommendations

From the discussion above it is clear that agents which cause large pH shifts can give false-positive results. The genotoxicity of 2,4-D, for example, may well be attributable to its acidic properties (Zetterberg, 1979). One approach to avoid potential problems might be to conduct all in vitro assays at neutral pH. The results of Morita et al. (1989b) indicate that neutralisation of the treatment medium may prevent pH-related genotoxicity, though these authors caution that this should only be done if the solubility or stability of the test chemical is not affected and hence any genotoxic property masked. However, this recommendation would imply that all compounds be tested over a range of pH values and this would markedly increase the burden of testing. Also, except for absorption through the stomach, human exposure is unlikely to involve non-physiological pH levels.

It is clear that the effect of a test chemical on the pH of the treatment medium should always be measured and it is recommended that positive results associated with pH shifts in the test system of greater than 1 unit should be viewed with caution and confirmed in experiments conducted at neutral pH.

6. Overall summary and recommendations

We have addressed the question of whether genotoxicity can be generated under extreme culture conditions which are irrelevant to the situation in vivo. Most of the available data relate to clastogenesis. Four in vitro conditions have been considered.

(a) Excessively high concentrations

Chromosome aberrations, TK mutations in L5178Y mouse lymphoma cells, morphological transformation, DNA-strand breakage in mammalian cells and mutations in yeast can be induced at very high concentrations of non-DNA reactive chemicals which cause a significant increase in the osmolality of the culture medium. To avoid this problem upper concentration limits for testing have been suggested, e.g. 10 mM in clastogenesis tests. However, some chemicals that are clastogenic in rodent bone marrow are only detectable in vitro at high concentrations, in part reflecting the inadequacies of metabolic activation systems. We conclude that if an upper concentration limit of 10 mM is adopted, with a rigorous protocol, very few in vivo clastogens will be missed and we recommend the use of a 10 mM limit for clastogenicity tests.

Of the 50% of tested chemicals which are clastogenic in vitro but not in vivo, probably less than 5% are clastogenic through osmolality effects.

Genotoxicity is not an inevitable consequence of exposure to high concentrations of chemicals.

Other mechanisms of indirect genotoxicity in addition to hypertonicity (e.g. enzyme inhibition, production of active oxygen species) may show true threshold responses at concentrations above those that can be achieved as a result of human exposure.

(b) High levels of cytotoxicity

Unlike the artefactual genotoxicity that occurs at high concentrations of some chemicals we have not obtained evidence for similar artefactual increases in gene mutations or chromosomal aberrations at high levels of cytotoxicity, although there is some evidence that double-stranded breaks in DNA may occur only at highly toxic levels after treatment with some chemicals. For mutation as-

says, the upper limits of cytotoxicity are determined by practical concerns such as limiting the variability that occurs at low survival. For chromosomal aberration assays we do not have a database to compare in vitro results at varying levels of toxicity with in vivo clastogenicity or carcinogenicity, but it appears that some chemicals that are clastogenic in vivo may be detected only at quite toxic concentrations in vitro. Further analyses are urgently required.

Relationships between genotoxicity and cytotoxicity in vitro can vary markedly depending on the endpoints studied, sampling time, exposure time and cell type; quantitative extrapolation to the in vivo situation must be undertaken with caution.

(c) Metabolic activation systems

Under certain conditions S9 mix may itself be genotoxic (e.g. chromosome aberrations in CHO cells, mutation in *E. coli* and mouse lymphoma cells). It is important that the underlying mechanism be elucidated because there may be chemicals whose genotoxicity in vitro is simply the result of inducing these conditions; this could lead to spurious positive results in genotoxicity testing. There is some evidence that active oxygen species produced *via* lipid peroxidation of microsomes are responsible. Certain endpoints and test systems appear to be less susceptible than others to S9 induced genotoxicity. It is probably premature to advocate modifications to current test procedures (e.g. addition of radical scavengers) until the mechanism is better understood.

(d) Extremes of pH

Extremes of pH can induce chromosome aberrations in mammalian cells, mutation in *E. coli* and mouse lymphoma cells and gene conversion in yeast but the mechanism is not clear. The effect of a test chemical on the pH of the medium should always be measured and positive results associated with pH shifts of greater than one pH unit should be viewed with caution and confirmed at neutral pH.

The artefactual genotoxicity associated with excessive concentrations, metabolic activation systems and pH extremes probably accounts for only a small part of the discrepancy between in vitro and in vivo results. Nevertheless, identification of these factors with appropriate modification of protocols should help to improve the credibility of in vitro tests in predicting in vivo response and, ultimately, human risk.

Acknowledgements

The Chairman (D.S.) is supported by the U.K. Cancer Research Campaign. ICPEMC wish to acknowledge the support of the U.K. Health and Safety Executive under contract No. 1/MS/126/272186.

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Respiratory health effects of long-term exposure to different chromium species in stainless steel production

M. Huvinen*, J. Uitti†, P. Oksa†, P. Palmroos† and P. Laippala‡
*Outokumpu Oyj, Espoo; †Tampere Regional Institute of Occupational Health and Clinic of Occupational Medicine, Tampere University Hospital, Tampere; and ‡School of Public Health, University of Tampere and Tampere University Hospital, Tampere, Finland

The aim of this study was to determine whether occupational exposure to chromite, trivalent chromium (Cr^{3+}) or hexavalent chromium (Cr^{6+}) causes respiratory diseases, an excess of respiratory symptoms, a decrease in pulmonary function or signs of pneumoconiosis among workers in stainless steel production. Altogether, 203 exposed workers and 81 referents with an average employment of 23 years were investigated for indicators of respiratory health on two occasions, in 1993 and in 1998. Data collection with a self-administered questionnaire, flow volume spirometry, measurement of diffusing capacity, chest radiography and laboratory tests were carried out by a mobile research unit. Exposure to different chromium species and other metals was monitored regularly and studied separately. No adverse respiratory health effects were observed in the group exposed to Cr^{6+}, either in comparison with the control group in the first cross-sectional study or during the additional 5 year follow-up. Among the Cr^{3+} exposed people, the production of phlegm, shortness of breath and breathlessness on exertion were significantly more frequent than in the control group, but the frequency of the symptoms did not increase during the follow-up; no differences were observed in the lung function tests and the radiographic findings did not progress. In the chromite group, the prevalence of breathlessness on exertion was higher than in the control group. However, in the follow-up, the occurrence of symptoms did not differ from 1993 to 1998. In the first study, most parameters of lung function were lower among the smokers in the chromite group than among the smoking controls, but in 1998 the difference was less marked. An average exposure time of 23 years in modern ferrochromium and stainless steel production and low exposure to dusts and fumes containing Cr^{6+}, Cr^{3+}, nickel and molybdenum do not lead to respiratory changes detectable by lung function tests or radiography. The workers exposed to Cr^{3+} had more respiratory symptoms than those in the control group. The workers in the chromite mine had lower lung function test results than the control group due to earlier exposure to higher dust concentrations.

Key words: Ferrochromium production; hexavalent chromium exposure; molybdenum exposure; nickel exposure; respiratory health; stainless steel production; trivalent chromium exposure.

Received 4 December 2001; revised 11 March 2002; accepted 30 April 2002

Correspondence to: Markku Huvinen, Outokumpu Oyj, PO Box 27, FIN-02201 Espoo, Finland. Tel: +358 9 4212450; fax: +358 9 4212120; e-mail: markku.huvinen@outokumpu.com

Introduction

The respiratory health effects of occupational exposure to chromium compounds have been studied in several

Occup. Med. Vol. 52 No. 4, pp. 203–212, 2002
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industries and occupations, but data on the chronic effects in stainless steel production and its different stages are limited. Chromium [1,2], chromium salts [3,4] and fumes of chromium trioxide [5] have been reported to cause asthma. Chromates, among other exposure agents in chromium plating, welding and ferrochromium production, have been connected with cases of occupational asthma [6]. Mining dust from chromite ore has been reported to cause chronic bronchitis [7]. A transient decrease in lung function has been found among chromium platers [8–10]. Increased prevalences of obstructive lung disorders were found among electrofurnace workers in a ferrochromium plant, possibly due to high levels of total dust, especially amorphous silica dust [11]. Controversial results have been reported on the risk of pneumoconiosis in chromate production [11–13]. No cases of pneumoconiosis were found among workers in a Norwegian ferrochromium plant [11].

Our previous study [14] indicated that long-term exposure to low levels of hexavalent chromium (Cr^{6+}) does not lead to any respiratory changes detectable by lung function tests or radiography, or to an increase in symptoms of respiratory disease. Among the workers exposed to trivalent chromium (Cr^{3+}), the production of phlegm, shortness of breath and breathlessness on exertion tended to appear more frequently than in the control group. The lung function test results were lower and the radiological findings were more frequent among the workers from the chromite mine than among the controls. These results were based on information from a cross-sectional study completed with information on industrial hygiene measurements of earlier exposure [14,15].

Thus, the aim of this study was to assess effects of long-term exposure to different species of chromium on the respiratory health of workers after 23 years of mean exposure. The same cohort as in the earlier study [14] was studied 5 years later. The study was also supplemented with information on exposure, including measurements of the current exposure levels to total chromium, Cr^{6+} and other air impurities in stainless steel production.

Methods

Process description

The integrated production chain of Outokumpu Oyj is unique; the mine and all stainless steel production plants are in the same region in northern Finland.

The chromite ore is mined from an open pit mine. The chromium oxide content of the ore is ~26%. After crushing, lump concentration, grinding, fine concentration and dewatering, the chromium oxide concentration rises to 42%.

In the production of ferrochrome, the fine concentrate is ground, mixed with bentonite as a binding agent, pelletized into 15 mm balls and hardened in the sintering furnace at 1400°C. Coke and quartz are added to the pellets and lump concentrate, and fed into the preheater. The heated material flows down to the arc furnace. The arc furnace is a fully closed system. In the furnace, chromite is reduced into metallic ferrochromium in the presence of carbon. Ferrochrome produced in two furnaces is tapped off at 2 h intervals. Molten ferrochrome is transported directly to the steel melting shop or cast into moulds made of finely divided ferrochrome.

In the steel melting shop, such raw materials as various types of scrap, both unalloyed and stainless, as well as alloying materials such as nickel and molybdenum, are combined with ferrochrome. These raw materials are melted in a closed arc furnace and blown with oxygen to keep the silicon content to the required level. The carbon content of the steel is lowered to the required level in an AOD (argon–oxygen decarbonization)-converter by blowing with argon and oxygen. At the end of this converting process, excess sulphur is removed with lime.

Next, in the continuous casting machine, the steel flows from the ladle via an intermediate holding basin to water-cooled copper moulds and then to a casting bow. After straightening, the slabs move to flame cutting, where they are cut to the lengths ordered.

In the next stage, the slabs are rolled in the hot rolling mill, which consists of a walking beam furnace, a roughing mill and a Steckel-type finishing mill.

On the coil build-up line of the cold rolling mill, the hot rolled strips are welded end to end to form long, uniform strips for the subsequent stages of the process. The annealing and pickling line, in which the strip is annealed to make its internal structure uniform and pickled to clean the surface, is formed by a series of units in the same line. The main parts are the gas-fired furnaces, the shot blasting units and the pickling units. Pickling is carried out electrolytically in a neutral Na_2SO_4 solution followed by mixed acid pickling (HNO_3 + HF), which ensures the cleanliness of the strip and gives it the proper stainless colour.

Cold rolling, in which the cold steel strip is reduced to its final thickness, is carried out in the Sendzimir rolling mills which operate on a backwards and forwards principle. The cold rolled strip is then lightly rolled in the skin pass mill to improve the flatness of the strip and finally split and cut to the ordered dimensions.

Subjects

All 316 workers who participated in a cross-sectional respiratory health study in 1993 [14] were invited to participate in this follow-up study. No cases of lung cancer or occupational asthma appeared among these workers during the follow-up. Two subjects who had left the company could not be contacted. Three workers had died from cardiac infarction. One subject could not be

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studied because of labile hypertension. Five workers had changed production departments between 1993 and 1995: one from an exposed group to the control group, and two from the exposed groups and two from the control group to departments outside this study (transportation and construction departments). These changes were not due to health problems. There were 16 workers who did not volunteer to participate and five workers who did not show up for the examinations. Thus, altogether, 284 workers (90%) finally participated (Table 1). The subjects were divided into the same four groups as in the previous study according to exposure to different chromium compounds: Cr^{6+} (furnace department of the ferrochromium plant and the steel melting shop); Cr^{3+} (sintering and crushing departments); chromite (chromite mine); and a control group. The control group consisted of workers from the cold rolling mill (the Sendzimir rolling mill, the skin pass mill, and the splitting and cutting line), because their level of exposure to chromium or dust in general was extremely low—or non-existent.

Exposure

An exposure study was carried out throughout the entire production chain in 1987 [15]. Since then, the exposure levels have been monitored on a continuous basis. Due to technological improvements in the production processes, the exposure levels have decreased during the last decade, especially in the steel melting shop, where the highest exposure levels to Cr^{6+} were observed in 1987 (Table 2).

In the furnace department of the ferrochromium plant (Cr^{6+} group), the average dust exposure was 1.5 mg/m^3 in 1987 [15]. The dust contained an average of 5–10% chromium. The proportion of Cr^{6+} of the total chromium was 0.1–0.3%. The highest concentrations were detected during tapping in the vicinity of the tap hole, where the proportion of Cr^{6+} was 10-fold (1–3%) the level in other areas.

In the steel melting shop (Cr^{6+} group), the median exposure to total dust was 1.8 mg/m^3 (0.70 mg/m^3 in 1999). The median Cr^{6+} concentration was 0.5 mg/m^3 (0.003 mg/m^3 in 1999). The highest values among personal samples were detected in the handling of molten metal by the arc furnace (6.6 mg/m^3 in 1978 and 0.0037 in 1999). Although, judged from the stationary samples (in which the amount of air collected was large), Cr^{6+} seemed to be present at low concentrations throughout the steel smelting shop, it exceeded the detection limit of 0.5 mg/m^3 in only some of the personal samples.

The mean postshift urinary concentration of chromium was 0.02 mmol/l for the 40 workers in the steel melting shop in 1999 (0.04 mmol/l in 1987 [15] and 0.03 mmol/l in 1993 [14]). The maximum concentration was 0.07 mmol/l (0.34 mmol/l in 1987 [15] and 0.08 mmol/l in 1993 [14]).

In the sintering and crushing departments (Cr^{3+} group), the average dust exposure was 2.4 mg/m^3. At the chromite mine in Kemi (chromite group), the average dust concentration was 1 mg/m^3. The median personal exposure to chromium was 22 mg/m^3; Cr^{6+} was not detected in any of the samples.

The total dust content was low throughout the cold rolling mill (control group), averaging 0.3–0.5 mg/m^3.

Table 1. Characteristics of the study groups in 1998

Characteristic	*Cr^{6+} group (n = 104)*	*Cr^{3+} group (n = 68)*	*Chromite group (n = 31)*	*Control group (n = 81)*
Age (years)				
Mean (SD)	48.0 (6.9)	49.9 (6.6)	48.8 (7.8)	45.6 (7.0)
Range	34–64	34–65	34–67	34–68
Height (cm)				
Mean (SD)	174.4 (6.0)	174.6 (5.4)	173.5 (6.5)	173.0 (7.2)
Range	160–190	161–186	161–189	156–187
Weight (kg)				
Mean (SD)	81.4 (10.7)	82.5 (10.8)	81.2 (12.1)	81.4 (10.9)
Range	56–111	64–110	62–110	60–110
Work history (years)				
Mean (SD)	21.0 (4.2)	25.5 (4.6)	23.8 (5.5)	19.4 (2.7)
Range	13–31	14–31	14–31	13–23
Smoking status (%)				
Non-smoker	34.6	27.9	16.1	32.1
Ex-smoker	38.5	39.7	51.6	24.7
Current smoker	26.9	32.4	32.3	43.2
Present smoking (cigarettes/day)				
Mean (SD)	18.2 (11.7)	18.7 (5.4)	17.3 (3.6)	15.9 (8.5)
Total duration of smoking (years)				
Mean (SD)	18.4 (10.4)	21.9 (11.2)	23.4 (10.0)	21.9 (9.9)

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Table 2. Exposure to total dust, chromium, hexavalent chromium, nickel and molybdenum in the steel melting shop

	Personal samples		*Stationary samples*	
	1987	*1999*	*1987*	*1999*
Total dust (mg/m^3)				
Median (*n*)	1.8 (153)	0.70 (12)	1.3 (63)	0.70 (34)
Maximum	18.0	2.50	7.9	3.30
Chromium (mg/m^3)				
Median (*n*)	0.0300 (82)	0.0047 (6)	0.0406 (34)	0.0053 (17)
Maximum	0.4320	0.0135	0.6030	0.0177
Hexavalent chromium (mg/m^3)				
Median (*n*)	0.0005 (72)	0.0003 (10)	0.0005 (28)	0.0030 (6)
Maximum	0.0066	0.0007	0.0037	0.0190
Nickel (mg/m^3)				
Median (*n*)	NA	0.0018 (6)	NA	0.0031 (17)
Maximum	NA	0.0094	NA	0.0300
Molybdenum (mg/m^3)				
Median (*n*)	NA	0.0003 (6)	NA	0.0006 (17)
Maximum	NA	0.0023	NA	0.0040

NA = not analysed.

In general, the content of chromium in the air in the cold rolling mill was lower than the detection limit of the measurement method.

Methods

A questionnaire was sent to the participants 1 week before the clinical examinations. It was based on the standardized questionnaire of a study carried out in the wool textile industry by the Edinburgh Study Group [16] and the definitions from the Medical Research Council (MRC) questionnaire [17] and it asked for information on personal characteristics, occupational history, respiratory symptoms, smoking habits, medication, and personal and family histories of allergic and pulmonary diseases. Some questions considered, among other things, the following items: cough, phlegm, shortness of breath and wheeze. Symptoms of rhinitis and eye irritation were also included.

Cough, lasting >3 months and improving after a holiday of >1 week, was considered to be work related. Similarly, dyspnoea occurring at least twice a month and caused or worsened by impurities in the work environment or during a work shift, but becoming better after a week's holiday, was regarded as work related. Questions about back or stomach pain and general health status were used as control questions, because they were not considered to be associated with the current occupational exposure.

Spirometry, measurement of diffusing capacity, a chest X-ray and laboratory tests were carried out by a mobile research unit with experienced laboratory technicians.

Spirometry was performed on each subject with a computerized flow volume spirometer (M905; Medikro Oy, Kuopio, Finland). Each subject was seated and wore a nose clip. At least three acceptable forced maximal expirations were performed according to the standards of the American Thoracic Society [18]. From the maximum expiratory flow volume curves, the highest forced vital capacity (FVC), forced expiratory volume in 1 s ($FEV_{1.0}$), flow rates at 50 and 25% of the vital capacity (MEF_{50}, MEF_{25}) and the mean flow (MMEF) were read. All of the values were also expressed as percentages of predicted values in Finland [19].

The spirometer was calibrated daily. Measurements were performed by two trained laboratory technicians. The technicians' performances of spirometry were compared before the study. The coefficient of variation in the FVC of the people tested was 0.74% and that of the $FEV_{1.0}$ was 1.37%.

Before the study, the M905 spirometer was compared with the M101 spirometer used in the previous study. (Both spirometers were manufactured by Medikro Oy, Kuopio, Finland.) The spirometers had a systematic difference. Results with the M905 model were slightly higher. Correction was made by reducing the 1998 FVC values by 3.7% and the $FEV_{1.0}$ values by 5.6%.

The diffusing capacity of the lungs for carbon monoxide (T_{LCO}) was measured with the Jaeger transfer test and a single-breath method. Although equipment was not the same as in the previous study, it was chosen because of the same techniques and gas concentrations. Alveolar volume (V_A) is the total lung capacity when the T_{LCO} is measured. The specific diffusion capacity is T_{LCO}/V_A. At least two satisfactory acceptable measurements were performed for each person. The mean value of the two nearest measurements was chosen [20]. The values were

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adjusted to the real-time haemoglobin measurement [21]. The results were expressed as percentages of predicted values in Finland [19].

Two trained laboratory technicians examined an equal number of people. Their measurement of diffusing capacity was tested before the study. The coefficient of variation in the results of these T_{LCO} measurements was 2.65%.

Diffusing capacity is a complex phenomenon and the detailed technology of different equipment varies. Diffusing capacity values were analysed cross-sectionally in 1998. Because of a change in the diffusing capacity equipment, there was no longitudinal comparison.

In the radiographic examination, a full-size, postero-anterior chest radiograph was taken. The radiographic examinations were performed only on workers in the exposed groups, not on those in the control group. The radiographs of each exposed subject from 1993 and 1998 were classified according to the modified classification of the International Labour Office (ILO) [22]. The radiographs of each subject were examined in random order with the identification labels covered. The progression was first estimated in a side-by-side comparison of the radiographs without information on the time sequence. Then, the later radiograph was classified separately by two radiologists with experience in ILO classification. Progression was recorded if the later radiograph was estimated (in a side-by-side comparison) to have more profusion of small opacities qualitatively than the first, even if the radiographs were classified into the same profusion category.

Statistical methods

Basic statistics were used to describe the data. The frequency tables were constructed for the prevalence of symptoms for a comparison between each exposure group and the control group, and they were analysed with Fisher's exact tests. Tables were set up for the non-smokers and smokers (including ex-smokers) separately, and also for the non-smokers and smokers combined. The effect of exposure on the pulmonary functions in the cross-sectional study was analysed with Student's *t*-tests. The results of both the smokers and the non-smokers were analysed.

In the follow-up study, the changes in symptoms were divided into three groups according to whether the symptom had appeared, remained unchanged or disappeared. The differences in the symptom changes between groups were analysed with χ^2 tests for the smokers and non-smokers separately. An analysis of covariance was used when the effect of exposure on pulmonary functions was studied over the follow-up time. In the analysis, the dependent variable was the change in pulmonary function and the independent variables were study group and smoking status (as a dichotomous variable), the lung function measurement in question in 1993 being used as a covariate. Age was not taken into account in the models because the analysed lung function values were the proportions of the absolute and reference values, for which the effect of age had already been eliminated. The limit for statistical significance was set at 0.05. The computation was carried out using Statistica/Win (v. '98) software.

Results

Subjects

The participation of the workers in this follow-up study was 91% for the exposed groups and 83% for the control group.

The study groups were similar in height and weight in 1993 and 1998 (Table 1). The same applies for the age distribution of the groups, the Cr^{3+} group being the oldest (mean 49.9 years) and the control group being the youngest (mean 45.6 years). During the follow-up, there was a change in the smoking habits of the chromite-exposed group in that 58.3% were current smokers in 1993, but only 32.3% were current smokers in 1998.

Respiratory symptoms

No difference was observed in the prevalence of respiratory symptoms between the group exposed to Cr^{6+} and the referents; nor was the change during the 5 year follow-up different for these two groups (Table 3).

In 1998, the production of phlegm, shortness of breath and breathlessness on exertion were significantly more frequent in the Cr^{3+} group than in the control group when the smokers and non-smokers were combined (Tables 4 and 5). Both the smoking and non-smoking Cr^{3+} workers tended to have a higher prevalence of all three respiratory symptoms than the workers in the control group, shortness of breath being statistically significantly more frequent among the non-smoking Cr^{3+} workers (4 out of 18 subjects) than among the non-smoking controls (none among 26 subjects). The prevalence of phlegm production was also higher among the smoking Cr^{3+} workers (31%) than among the smoking controls (13%). In the follow-up, there were no differences in the changes in the occurrence of symptoms from 1993 to 1998 when the Cr^{3+} group was compared with the control group, except for the occurrence of rhinitis, which was observed to decrease significantly more ($P = 0.01$) among the non-smoking Cr^{3+} workers than among the non-smoking controls.

The prevalence of breathlessness on exertion tended to be higher in the chromite group than in the control group ($P = 0.07$). The change in the prevalence of any symptom over time did not differ between the chromite group and the controls.

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Table 3. Differences in percentages [with 95% confidence intervals (CI)] within exposure groups in 1993 and 1998, and differences in percentages (with 95% CI) between the exposure groups and the control group in 1998

	Cr^{6+} group (n = 104)		*Cr^{3+} group (n = 68)*		*Chromite group (n = 31)*		*Control group (n = 81)*
Symptoms	*Difference within group (CI)*	*Difference between group and control (CI)*	*Difference within group (CI)*	*Difference between group and control (CI)*	*Difference within group (CI)*	*Difference between group and control (CI)*	*Difference within group (CI)*
Cough >3/12 months	1.9 (−6.5–10.3)	−1.1 (−10.3–8.1)	7.3 (−5.8–20.4)	3.3 (−8.0–14.6)	−1.4 (−18.9–16.1)	4.0 (−10.8–18.8)	−1.7 (−11.2–7.8)
Work-related cough	3.9 (−1.3–9.1)	−0.6 (−4.9–3.7)	4.4 (−4.7–13.5)	3.4 (−3.2–10.0)	−3.3 (−14.0–7.4)	4.0 (−5.3–13.3)	−1.3 (−5.4–2.8)
Phlegm >3/12 months	7.7 (−1.5–16.9)	−1.5 (−10.4–7.4)	0.0 (−15.1–15.1)	16.8 (4.1–29.5)	6.5 (−13.1–26.1)	5.0 (−9.6–19.6)	−3.7 (−12.6–5.2)
Shortness of breath (and wheezing)	2.9 (−3.7–9.5)	1.0 (−4.9–6.9)	−1.7 (−14.4–11.0)	14.1 (4.0–24.2)	6.0 (−9.0–21.0)	3.1 (−7.0–13.2)	2.4 (−4.3–9.1)
Breathlessness on exertion[a]	−0.9 (−9.4–7.6)	5.2 (−2.9–13.3)	−1.7 (−14.8–11.4)	13.1 (2.2–24.0)	−6.5 (−24.7–11.7)	13.1 (−1.8–28.0)	−1.4 (−8.5–5.7)
Rhinitis >3/12 months	−7.7 (−20.1–4.7)	0.4 (−13.3–14.1)	19.2 (3.2–35.2)	−5.4 (−20.2–9.4)	−3.3 (−26.2–19.6)	−1.0 (−20.4–18.4)	−8.6 (−22.5–5.3)
Eye symptoms >3/12 months	−0.9 (−11.5–9.7)	5.6 (−5.0–16.2)	3.0 (−8.9–14.9)	−0.4 (−11.4–10.6)	−25.8 (−42.9—8.7)	15.4 (−2.2–33.0)	−2.5 (−12.6–7.6)

[a]Shortness of breath when hurrying on level ground.

Table 4. Prevalence (%) of respiratory symptoms in non-smokers

	Cr^{6+} group (n = 41)		*Cr^{3+} group (n = 21)*		*Chromite group (n = 5)*		*Control group (n = 27)*	
Symptoms	*1993*	*1998*	*1993*	*1998*	*1993*	*1998*	*1993*	*1998*
Cough >3/12 months	12.8	8.1	9.5	0.0	0.0	0.0	3.7	8.3
Work-related cough	7.3	0.0	9.5	0.0	0.0	0.0	3.7	3.7
Phlegm >3/12 months	14.6	4.9	23.8	19.1	0.0	0.0	3.7	7.4
Shortness of breath (and wheezing)	2.4	7.3	9.5	20.0*	20.0	0.0	3.7	0.0
Breathlessness on exertion[a]	9.8	9.8	4.8	14.3	0.0	0.0	0.0	3.7
Rhinitis >3/12 months	26.8	36.6	57.1	28.6	0.0	0.0	18.5	29.6
Eye symptoms >3/12 months	29.3	24.4	14.3	19.1	0.0	20.0	7.4	14.8

[a]Shortness of breath when hurrying on level ground.

*$P < 0.05$, exposure group versus control group in Fisher's exact test (differences in 1993 not analysed).

Table 5. Prevalence (%) of respiratory symptoms in smokers (current and former)

	Cr^{6+} group (n = 63)		*Cr^{3+} group (n = 47)*		*Chromite group (n = 26)*		*Control group (n = 52)*	
Symptoms	*1993*	*1998*	*1993*	*1998*	*1993*	*1998*	*1993*	*1998*
Cough >3/12 months	10.0	9.8	26.1	19.1	15.4	17.4	11.5	11.9
Work-related cough	4.8	3.2	10.6	8.5	3.9	7.7	0.0	1.9
Phlegm >3/12 months	19.1	12.7	29.8	31.9*	26.9	19.2	9.6	13.5
Shortness of breath (and wheezing)	11.1	3.2	19.2	17.0	11.5	8.0	7.7	5.9
Breathlessness on exertion[a]	11.1	12.7	23.4	21.7	15.4	23.1	7.7	8.0
Rhinitis >3/12 months	25.4	31.8	42.6	27.7	34.6	38.5	26.9	36.5
Eye symptoms >3/12 months	11.1	15.9	17.0	10.6	3.9	30.8	13.5	13.5

[a]Shortness of breath when hurrying on level ground.

*$P < 0.05$, exposure group versus control group in Fisher's exact test (differences in 1993 not analysed).

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Lung function tests

Among the smokers (including ex-smokers) and the non-smokers, no exposure-related deterioration in lung function was observed in the group exposed to Cr^{6+}, or in the group exposed to Cr^{3+} and the controls, nor did the change during the 5 year follow-up differ for these groups. In general, the smokers had lower lung function test results (Tables 6 and 7). The diffusing capacity and specific diffusing capacity were significantly higher among the smokers in the Cr^{6+} group than among the smoking controls.

Among the smokers in the chromite group, FVC was significantly lower ($P = 0.03$) than among the smoking controls in the cross-sectional comparison in 1998. No differences in $FEV_{1.0}$, MEF_{50}, MEF_{25}, MMEF or peak expiratory flow (PEF) were seen in the comparison between each exposed group and the control group.

Because of the small number of non-smoking workers in the chromite group, their results could not be analysed separately. When the smokers and non-smokers in the chromite group were combined and the group was compared as a whole, no significant differences between the exposed workers and the controls were observed for any of the lung function variables. In the follow-up study, $FEV_{1.0}$ decreased less in the chromite group than in the control group.

Radiographs

In the comparison of the radiographs from 1998 with those from 1993, the profusion of small opacities had progressed in three people and the profusion category for these workers was ≥1/0. One of these workers was exposed to Cr^{6+}, one belonged to the chromite group and one was exposed to Cr^{3+}.

Discussion

The aim of both studies (the cross-sectional in 1993 and follow-up in 1998) focused on assessing the long-term respiratory effects of exposure to different chromium compounds in the production chain of stainless steel. The

Table 6. Lung function measurements [mean (SD)] as a percentage of predicted values (non-smokers)

Lung function variable	*Cr^{6+} group (n = 41)*		*Cr^{3+} group (n = 21)*		*Chromite group (n = 5)*		*Control group (n = 27)*	
	1993	*1998*	*1993*	*1998*	*1993*	*1998*	*1993*	*1998*
FVC	96.4 (11.1)	94.2 (12.0)	93.4 (9.1)	93.6 (11.0)	96.1 (11.1)	97.0 (12.3)	93.4 (9.7)	92.4 (8.5)
$FEV_{1.0}$	95.5 (11.4)	91.9 (11.3)	92.9 (11.2)	91.2 (12.9)	94.2 (10.8)	94.3 (13.2)	94.7 (11.6)	92.3 (10.5)
FEV%	99.8 (6.6)	97.9 (7.2)	99.1 (6.7)	97.2 (7.4)	97.7 (6.6)	97.4 (8.4)	101.5 (5.8)	99.8 (5.8)
TLCO	104.8 (11.8)	112.1 (13.9)	107.8 (15.8)	115.5 (16.9)	105.6 (5.9)	109.7 (4.8)	106.6 (14.7)	112.1 (11.7)
TLCOHb	104.1 (11.9)	109.2 (13.9)	107.8 (14.7)	110.6 (19.4)	105.9 (5.3)	106.1 (3.4)	106.3 (13.9)	107.4 (13.1)
TLCO/VA	108.6 (16.7)	109.9 (14.4)	113.2 (17.3)	112.0 (15.8)	111.4 (21.3)	104.0 (12.7)	111.5 (18.2)	110.9 (13.9)
TLCO/VAHb	108.0 (16.7)	107.2 (13.4)	113.3 (15.9)	107.1 (17.2)	111.4 (19.4)	101.0 (13.5)	111.4 (18.1)	106.0 (13.8)

Abbreviations: FVC = forced vital capacity; $FEV_{1.0}$ = forced expiratory volume in 1 s; FEV% = $FEV_{1.0}$/FVC × 100; TLCO = diffusing capacity; TLCOHb = diffusing capacity adjusted for haemoglobin; TLCO/VA = specific diffusing capacity; TLCO/VAHb = specific diffusing capacity adjusted for haemoglobin.

Table 7. Lung function measurements [mean (SD)] as a percentage of predicted values (smokers[a])

Lung function variable	*Cr^{6+} group (n = 63)*		*Cr^{3+} group (n = 47)*		*Chromite group (n = 26)*		*Control group (n = 52)*	
	1993	*1998*	*1993*	*1998*	*1993*	*1998*	*1993*	*1998*
FVC	93.5 (9.6)	89.6 (11.5)	90.7 (9.6)	90.3 (10.6)	87.8 (10.9)	87.2* (10.3)	94.9 (10.8)	92.9 (11.5)
$FEV_{1.0}$	91.8 (12.5)	87.9 (14.1)	89.1 (10.7)	87.6 (12.2)	85.1 (13.0)	83.0 (12.4)	92.6 (12.3)	88.5 (13.6)
FEV%	98.2 (8.0)	97.8 (7.8)	98.0 (7.1)	97.1 (7.8)	94.4 (8.1)	95.2 (8.4)	97.4 (7.2)	95.2 (8.7)
TLCO	98.5 (16.6)	109.0* (17.5)	92.4 (12.8)	102.9 (14.9)	93.1 (13.9)	100.3 (15.7)	94.9 (13.1)	102.1 (11.8)
TLCOHb	97.5 (15.5)	103.8* (16.6)	92.5 (12.6)	96.2 (14.6)	92.9 (13.7)	92.9 (16.3)	94.6 (13.6)	95.9 (11.8)
TLCO/VA	104.9 (16.56)	92.1* (13.0)	98.9 (15.9)	87.3 (14.1)	103.8 (15.9)	90.0 (16.4)	99.9 (14.6)	86.3 (10.8)
TLCO/VAHb	104.7 (16.4)	101.9* (14.4)	99.2 (15.9)	94.8 (16.1)	103.8 (16.3)	96.9 (19.6)	99.8 (15.2)	94.1 (12.5)

For abbreviations, see Table 6.

[a]Smokers include both current and ex-smokers.

*$P < 0.05$. Cr^{6+}, Cr^{3+} and chromite groups versus the control group (differences in 1993 not analysed).

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results of this follow-up study did not differ from those of our previous cross-sectional study 5 years earlier. Because the cross-sectional study design always involves risk of selection bias, we wanted to complete it with this follow-up in order to gather more accurate information on the risks for respiratory disorders. Even with the follow-up, it was not possible to remove the effect of potential bias developing from earlier time periods of exposure. It was, however, possible to demonstrate possible current effects of exposure to different chromium compounds on the respiratory system, if they exist. The group sizes were fairly small, especially the one from the chromite mine. All the workers meeting the inclusion criteria were, however, examined. Therefore, from the point of view of occupational health care, the follow-up increased the power in assessing risks for this population. The duration of employment in the exposed groups was sufficiently long for this purpose, the mean exposure period varying from 21 to 26 years. As with the majority of the labour force in general, all the workers in this study had been employed by the same production departments throughout their careers and their occupational exposure has been regularly monitored, which is an advantage for this kind of study design.

The control group was originally chosen from the same company, which ensured a similar socio-economic background and similar employment criteria for the groups. The alternative choice for a control group would have been controversial, in that a small town does not offer many workplaces with the same characteristics as the study population and most 'blue collar' workers have notable exposures to other pollutants that may disturb the comparison of exposed and 'unexposed' groups. Even clerical controls may have entered office work because of allergies and other selective health reasons. Although our controls were exposed to minor amounts of air pollutants in their work environment, it is very likely, according to accurate exposure measurements, that their exposure was not able to induce chronic respiratory effects. The follow-up study concentrated on comparing the change or stability of the differences in the variables between different groups with different exposure and, therefore, the long-term effects should have been apparent in this comparison, if such effects existed.

The participation rate was high. Altogether, 90% of those studied in 1993 participated in the follow-up study. The rate for the workers able and eligible to participate was, however, even higher, at 95%.

The health history of the former workers and the reasons for leaving the company were assessed by a questionnaire in 1993, and by personal inquiry and information from the medical records of the company in 1998. Thus, survivor bias can be regarded as small in this study.

We assessed the results (1993) of people who did not participate in the follow-up in 1998 (n = 32). For those from the Cr^{6+}, Cr^{3+} and control groups, there was a similar distribution of symptoms and mean lung function values. However, in the chromite group, three people had lung functions below the predicted values. This finding had to be taken into account in the interpretation of the results of the chromite group because of the small number of workers in that group.

According to biological monitoring, and stationary and personal exposure measurements, occupational exposure to chromium has been decreasing at the plant since the 1980s. The exposure levels of other alloying metals, such as nickel and molybdenum, are also low.

In a field emission scanning electron microscope analysis, the aerosols from the ferrochromium smelter were observed to contain agglomerates of particles with a diameter <1 mm [23]. Chromium seemed to be dissolved in a silica matrix. Most particles were covered with a thin layer of zinc oxide. The particles in the aerosols encountered in the steel melting shop were predominantly metal alloys. No pure chromium or nickel particles were observed. The particles had an iron oxide or iron core surrounded by chromium and nickel as alloys and silicates and oxides. These complexes are chemically tightly bound and insoluble in a biological environment.

The low level of metal fumes and the surface structure of the metal aerosols encountered in the workplace air in the ferrochromium smelter and the stainless steel melting shop may partly explain the scarcity of new respiratory findings.

Rather than being directed towards finding new cases of asthma or other diseases, the methods of our study were directed towards the early detection of worsening respiratory health (symptoms or subclinical lung function changes or radiological findings). The occupational health care unit of the company was able to collect data on diseases comprehensively at the individual level. No new cases of asthma, cancer or pneumoconiosis were found in 1993–1998.

The prevalences of symptoms were similar in 1993 and 1998. The workers in the Cr^{3+} group still had more symptoms (production of phlegm, shortness of breath and breathlessness on exertion) than the workers in the control group. The difference was significant in the group comparison in both years, without stratification or analysis according to known confounders. In 1993, the logistic regression analysis adjusted with confounders showed no difference in these prevalences between these groups. Therefore, because of the small number of observations, it was best to consider changes in the occurrence of symptoms and a group comparison, as we have described in the Statistical methods section. It showed that this excess of symptoms in the Cr^{3+} group was not related to smoking habits. The increased prevalence of the pattern of symptoms in the Cr^{3+} group seemed to be consistent in that

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it was also observed in 1993. Because there were no differences in the deterioration of lung functions between the Cr^{3+} group and the control group, this observation of respiratory symptoms does not indicate the parallel development of chronic obstructive pulmonary disease (COPD). The reason for this observation of excess of respiratory symptoms may be irritation, but the occupational exposure pattern does not lead to any evident explanation.

The $FEV_{1.0}$ decreased significantly less in the chromite group than in the control group. Other lung function variables also had the same tendency. The lung functions were, or tended to be, lower in the chromite group than in the control group both in 1993 and in 1998. However, the analysis of the changes in lung function was adjusted for the initial lung function value (as covariate) and therefore the lower starting point did not explain this result concerning change. The relative improvement of the lung function test in the chromite-exposed group was probably related to the decrease in smoking. The number of ex-smokers had doubled between 1993 and 1998 and, in the analysis, smokers and ex-smokers were not separated. However, it is likely that the causative agent behind the lower lung function tests in this group, also observed in 1993, is the exposure more than two decades ago to higher dust concentrations, including fibrous components [14].

The stability of the radiological findings indicates that the recent exposure levels to potentially fibrogenic dusts have been low. Radiological parenchymal findings according to the ILO classification were not common in a previous study [14]. Based on our radiological findings in two studies, it seems evident that exposure to chromium compounds at the levels described does not produce fibrosis. Selection bias is not an explanation for this observation, because it is unlikely that symptomless mild fibrosis would cause selection among exposed workers.

In conclusion, an average exposure time of 23 years in modern ferrochromium and stainless steel production with low exposure to dusts and fumes containing hexavalent and trivalent chromium, nickel and molybdenum does not lead to respiratory changes detectable by lung function tests or radiography. A slight increase in respiratory symptoms without deterioration of lung function was observed among the workers exposed to Cr^{3+}. The workers at the chromite mine had lower lung function test results than the control group, probably because of earlier exposure to higher dust concentrations.

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Occupational and Environmental Medicine 1996;53:741–747

Respiratory health of workers exposed to low levels of chromium in stainless steel production

Markku Huvinen, Jukka Uitti, Anders Zitting, Pekka Roto, Kristina Virkola, Pertti Kuikka, Pekka Laippala, Antero Aitio

Abstract

***Objectives*—To determine whether occupational exposure to chromite, trivalent chromium, or hexavalent chromium causes respiratory diseases, an excess of respiratory symptoms, a decrease in pulmonary function, or signs of pneumoconiosis among workers in an integrated chain of stainless steel production.**

***Methods*—This cross sectional study was carried out in 1993 and the inclusion criterion was a minimum of eight years of employment in the same production department. A self administered questionnaire was collected, and spirometry, measurement of diffusing capacity, chest radiography, and laboratory tests were carried out by a mobile research unit.**

***Results*—There were 221 workers in the exposure groups and 95 in the control group. The average duration of employment was 18 years. No significant differences in the odds ratios (ORs) of the symptoms were found between the exposure and the control groups. In a logistic regression analysis age and smoking significantly explained the occurrence of most of the respiratory symptoms. The smokers in the chromite group had significantly lower forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), and diffusing capacity than the corresponding values of the control group. The analysis of variance between study groups, smoking, and exposure time, without modelling for interactions, showed that the chromite group had lower values for FVC, FEV_1, and diffusing capacity than the other groups. The occurrence of small opacities was more frequent on the chest radiographs of the workers in the chromite group.**

***Conclusions*—An average exposure time of 18 years in ferrochromium and stainless steel production and exposure to dusts containing low concentrations of hexavalent or trivalent chromium do not lead to any respiratory changes detectable by lung function tests or radiography nor to any increase in symptoms of respiratory diseases. The lung function values were lower and the occurrence of radiological findings was more frequent among the workers from the chromite mine than among the controls. The difference was partly caused by differences in age and smoking habits, but evidently also partly by higher exposures more than two decades ago or by the fibrous components of the dust.**

(*Occup Environ Med* 1996;53:741–747)

Keywords: stainless steel production; chromium exposure; respiratory health

Outokumpu Oy, Espoo, Finland
M Huvinen

Tampere Regional Institute of Occupational Health, Tampere, Finland
J Uitti
P Roto

Institute of Occupational Health, Helsinki, Finland
A Zitting
A Aitio

Helsinki University Hospital, Helsinki, Finland
K Virkola

Outokumpu Polarit Oy, Tornio, Finland
P Kuikka

School of Public Health, University of Tampere and Tampere University Hospital, Tampere, Finland
P Laippala

Correspondence to: Dr Markku Huvinen, Outokumpu Oy, PO Box 27, FIN-02201, Espoo, Finland.

Accepted 11 July 1996

Chromate dust and fumes of chromium trioxide have been reported to cause asthma.[1] Chromates, among other exposure agents in chromium plating, welding, and ferrochromium production, have been connected with cases of occupational asthma[2] or bronchitis. Mining dust in an underground chromite ore mine has also been reported to cause chronic bronchitis.[3]

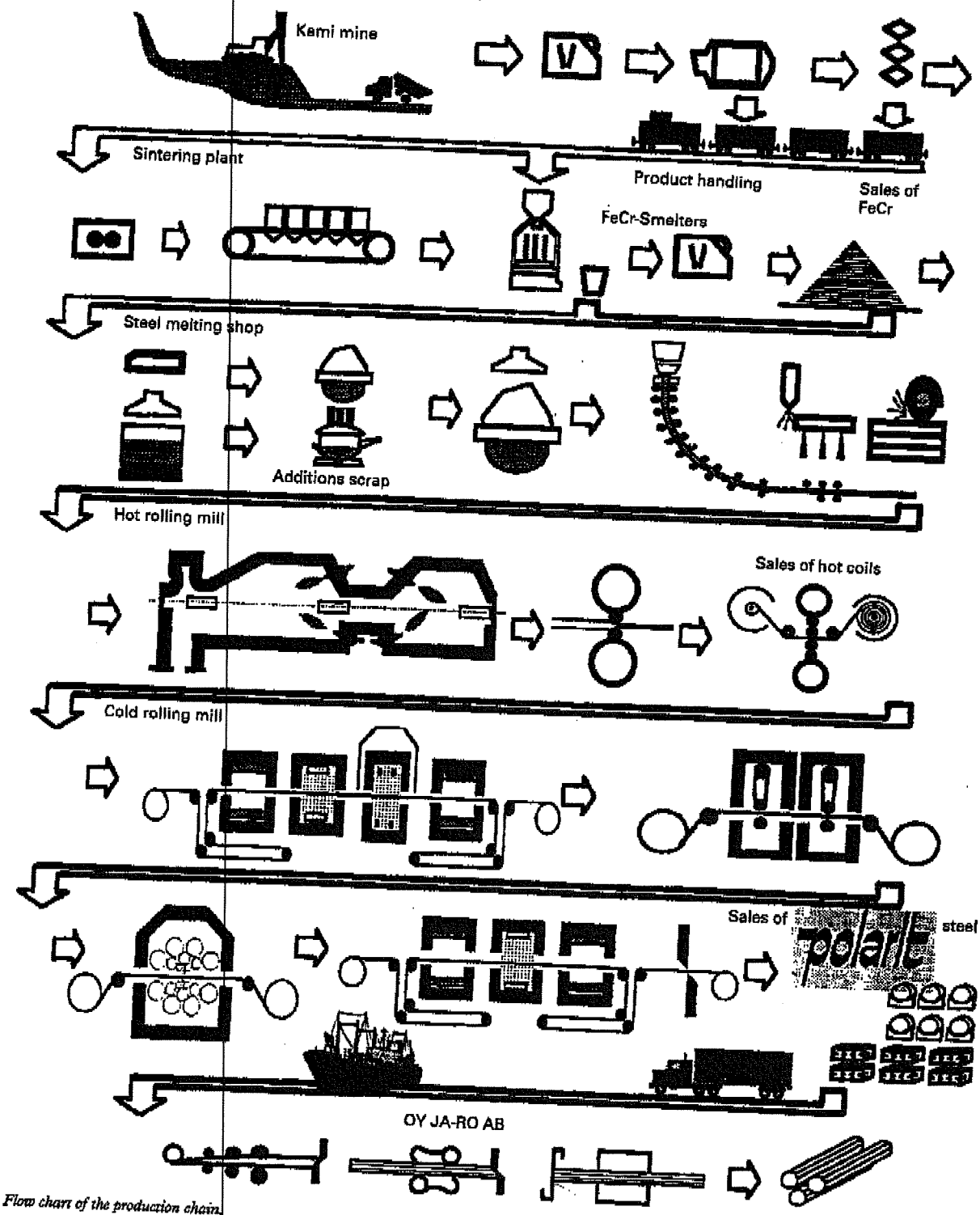
Obstructive effects on lung function have also been found among chromium workers.[4-6] In one study reduced forced vital capacity (FVC) and an increased prevalence of obstructive lung diseases were found among electrofurnace workers in a ferrochromium plant.[7] The author suggested that the effects were due to high levels of total dust, especially amorphous silica dust.

Nodular pneumoconiosis is another finding among workers in chromate production.[8 9] However, it has not been confirmed in some studies.[7 10] In a study on respiratory symptoms among 60 ferrochromium workers in Norway pneumoconiosis was diagnosed from the radiographical examinations.[7]

The integrated production chain of Outokumpu Steel Oy is unique; the mine and all the stainless steel manufacturing plants are in the same region. The figure shows a flow chart of the process. Unlike the rest of the ferrochromium furnaces in the world, the Outokumpu process is a fully closed system.

In an animal experiment[11] chromite particles from Outokumpu's chromium mine were considered to be fibrogenetically inert.

The purpose of the present study was to determine whether long term occupational exposure to low levels of chromite, trivalent chromium (Cr^{+3}), or hexavalent chromium (Cr^{+6}) causes respiratory diseases, an excess of respiratory symptoms, a decrease in pulmonary function, or signs of pneumoconiosis among workers in an integrated chain of stainless steel production, and to investigate whether Cr^{+3} and Cr^{+6} compounds (known to differ in their toxicity) also differ from each other in this respect.



Flow chart of the production chain.

Subjects and methods

SUBJECTS

At the time of the study, from March to May 1993, there were 892 workers in the production departments. The subjects were divided into four groups according to exposure to different chromium compounds: exposure to Cr^{+6}, Cr^{+3}, chromite, and a control group. In this study the inclusion criterion was a minimum of eight years of employment in the same department. Altogether 222 workers met the criteria of exposure and duration of employment and were placed in one of the three exposure groups. One of the workers did not agree to participate in the study. The control group consisted of workers from the cold rolling mill (the Sendzimir rolling mill, the skin pass mill, and the splitting and cutting

line), because their level of exposure to chromium or dust in general was extremely low—or non-existent. Out of the 132 potential controls, 95 agreed to participate. The lower response rate among controls was mainly due to the relative inconvenience to the workers in matching workshifts with study appointments. The total number of subjects was therefore 316 and all of them were men with similar ethnic and socioeconomic backgrounds.

The company also provided a list of names of the workers who had been working for at least eight years in any of the production departments under study and then resigned. There were 53 such workers; 17 of them had been exposed to Cr^{+6}, four to Cr^{+3}, and 19 to chromite; 13 of them had been working in the cold rolling mill where the control group worked. A questionnaire was sent to all of these former workers, but no clinical examinations were made.

EXPOSURE

An exposure study was carried out through the whole production chain in 1987.[12] At the chromite mine in Kemi (chromite group) the average dust concentration was 1 mg/m³. The median personal exposure to chromium was 22 μg/m³; Cr^{+6} was not detected in any of the samples.

In the furnace department of the ferrochromium plant (Cr^{+6} group) the average dust exposure was 1·5 mg/m³. The dust contained an average of 5%–10% chromium. The proportion of Cr^{+6} of the total chromium was 0·1%–0·3%. The highest concentrations were detected during tapping in the vicinity of the tap hole, where the proportion of Cr^{+6} was 10-fold (1%–3%) the level in other areas. In the sintering and crushing departments (Cr^{+3} group) the average dust exposure was 2·4 mg/m³.

In the steel smelting shop (Cr^{+6} group), the average exposure to total dust was 1·8 mg/m³. The dust contained 2%–4% chromium. The median Cr^{+6} concentration was 0·5 μg/m³. The highest values among personal samples were detected in the handling of molten metal by the arc furnace (6·6 μg/m³). Although judged from the stationary samples (in which the amount of air collected was large), Cr^{+6} seemed to be present at low concentrations throughout the steel smelting shop; it exceeded the detection limit of 0·5 μg/m³ in only some of the personal samples.

The total dust content was low throughout the cold rolling mill (control group); it averaged 0·3–0·5 mg/m³. In general, the content of chromium in the air in the cold rolling mill was lower than the detection limit of the measurement method.

METHODS

A questionnaire was sent to the participants one week before the clinical examinations. It was based on the standardised questionnaire of a study made in the wool textile industry by the Edinburgh Study Group[13] and the definitions from the Medical Research Council (MRC) questionnaire,[14] and it asked for information on personal characteristics, occupational history, respiratory symptoms, smoking habits, medication, and personal and family histories of allergic and pulmonary diseases. Some questions considered, among other things, the following items: cough, phlegm, shortness of breath, and wheeze. Symptoms of rhinitis and eye irritation were also included.

Cough, lasting more than three months and improving after a holiday of more than one week, was considered to be work related. Similarly, dyspnoea occurring at least twice a month and caused or worsened by impurities in the work environment or during a workshift, but becoming better after a week's holiday, was regarded as work related. Questions about back or stomach pain and general health status were used as control questions because they were not considered to be associated with the current occupational exposure.

Spirometry, measurement of diffusing capacity, a chest x ray film examination, and laboratory tests were carried out by a mobile research unit with two experienced laboratory technicians.

Spirometry was performed on each subject with a computerised flow volume spirometer ME 101 (Medikro Oy, Kuopio, Finland). The spirometer was calibrated each day with a 5 litre syringe. At least three satisfactory acceptable forced maximal expirations were performed according to the standards of the American Thoracic Society,[15] and all volumes were corrected to body temperature, pressure, and saturation (BTPS). Each subject was seated wearing a nose clip. From the maximum expiratory flow volume curves the highest FVC, forced expiratory volume in one second (FEV_1), and flow rates at 50% and 25% of the vital capacity (MEF_{50}, MEF_{25}) and their mean flow (MMEF) were read.

All of the values were also expressed as percentages of predicted values in Finland.[16] Two trained laboratory technicians examined an equal number of people. The technicians' performance of spirometry was compared before the study. The mean difference in the FVC of the people tested was minimal (2·1%).

Diffusing capacity of the lungs for carbon monoxide (TLCO) was measured with the Morgan transfer test and the single breath method. Alveolar volume (V_A) is the total lung capacity found at the time when the TLCO is measured. The specific diffusing capacity is TLCO/VA. The same laboratory technician performed at least two successful consecutive measurements for each person; the mean value of the two nearest test results was chosen.[17] These values were adjusted to the real time haemoglobin measurement.[18] The results were also expressed as percentages of predicted values in Finland.[16]

In the radiographic examination full size 35 × 43 cm x ray films were used. The radiographs were classified according to the modified classification system of International Labour Organisation (ILO).[19]

Two radiologists, both of whom were experienced with the ILO system, classified the radiographs individually without knowing the

Table 1 Characteristics of the study groups

Characteristic	Cr^{+6} group (n = 109)	Cr^{+3} group (n = 76)	Chromite group (n = 36)	Control group (n = 95)
Age (y):				
Mean (SD)	43·3 (6·9)	45·5 (6·8)	44·6 (7·6)	40·7 (7·1)
Range	29–59	29–60	30–63	30–64
Height (cm):				
Mean (SD)	174·6 (5·9)	174·8 (5·2)	174·1 ·6·3)	174·3 (6·9)
Range	159–189	163–187	162–190	157–191
Weight (kg):				
Mean (SD)	89·9 (10·7)	81·0 (11·2)	79·1 (13·5)	80·0 (11·8)
Range	61–110	57–108	54–117	61–140
Work history (y):				
Mean (SD)	16·0 (4·6)	20·8 (4·5)	19·5 (5·6)	14·4 (2·7)
Range	8–26	9–27	9–27	8–18
Smoking status (%):				
Non-smoker	37·6	29·3	16·7	35·1
Ex-smoker	33·4	32·0	25·0	19·2
Current smoker	28·4	38·7	58·3	45·7
Present smoking:				
Cigarettes/day:				
Mean (SD)	16·7 (6·5)	16·9 (5·4)	17·0 (5·4)	15·1 (7·2)
Total duration of smoking (y):				
Mean (SD)	16·3 (7·9)	18·9 (9·3)	20·1 (8·3)	17·4 (8·3)

names or exposure data of the subjects. If the classifications of the radiologists differed, the higher classification was recorded as the result. One of the radiologists was a certified NIOSH B reader. The radiographs of the control group were mixed with those of the exposed groups.

The urinary concentration of chromium was measured from the specimens of 44 workers from the steel smelting shop to ascertain the exposure level compared with the level in an earlier study in the same production chain.[12] Every other worker in the steel smelting shop was selected from the alphabetical payroll list. The specimens were collected in the afternoon after the workshift.

*Table 2 Prevalence of symptoms**

Symptom	Cr^{+6} group (n = 109) %	Cr^{+3} group (n = 76) %	Chromite group (n = 36) %	Control group (n = 95) %
Cough > 3/12 months	10·4	21·1	19·4	10·5
Work related cough	5·6	9·2 P = 0·01	2·8	1·1
Phlegm > 3/12 months	18·5	26·7 P = 0·01	25·0 P= 0·04	10·6
Shortness of breath (and wheezing)	8·3	14·5	11·1	6·3
Work related cough or shortness of breath	11·9 P = 0·04	13·2 P = 0·03	5·6	4·2
Breathlessness on exertion†	11·0	19·7 P = 0·03	13·9	8·4
Rhinitis > 3/12 months	26·2	46·1	30·6	29·1
Eye symptoms > 3/12 months	17·8	17·3	5·6	11·6
Abdominal pain	50·0	41·1	25·0	36·6
Back pain	64·5	65·8	50·0	60·2
Health condition:				
Good	43·1	48·7	52·8	60·0 P = 0·02
Average	49·5 P = 0·05	38·2	36·1	35·8
Poor	7·3	13·2 P = 0·03	8·3	4·2

*Cr^{+6}, Cr^{+3}, and chromite groups v control group, Student's two tailed *t* test.
†Shortness of breath when hurrying on level ground.

Table 3 Logistic regression analysis of respiratory symptoms

Symptom	Cr^{+6} group (n = 109) OR* (95% CI)	Cr^{+3} group (n = 76) OR* (95% CI)	Chromite group (n = 36) OR* (95% CI)
Cough > 3/12 months	1·12 (0·43–2·92)	1·61 (0·54–4·81)	1·22 (0·36–4·09)
Phlegm > 3/12 months	2·18 (0·80–5·92)	2·17 (0·70–6·76)	1·26 (0·37–4·31)
Shortness of breath	1·55 (0·49–4·91)	2·77 (0·76–10·08)	1·82 (0·42–7·94)
Chest tightness	1·19 (0·53–2·65)	1·39 (0·53–3·62)	1·24 (0·41–3·74)
Breathlessness on exertion†	1·25 (0·44–3·53)	1·92 (0·57–6·43)	0·96 (0·23–3·91)
Rhinitis	1·02 (0·49–2·12)	2·22 (0·92–5·33)	1·43 (0·49–4·18)
Any of these symptoms	1·39 (0·70–2·80)	2·11 (0·92–4·83)	1·11 (0·42–2·92)

*Age and duration of exposure as continuous variables and earlier pulmonary diseases as classified variables.
†Shortness of breath when hurrying on level ground.

STATISTICAL METHODS

Basic statistics were used to describe the data. The frequency tables were analysed with χ^2 statistics. The multivariate analysis was based on logistic regression analysis, where we have included possible confounders as predictors and their effect was adjusted, when needed. The effect of risk factors in the model were shown with adjusted odds ratios. The natural confounders in our study were age, exposure time, smoking, earlier lung disorders, and atopic diseases. When the relation between pulmonary functions and exposure were studied, we used Student's *t* test and analysis on covariate. Here the possible effect of confounders was taken into account using covariates. The possible confounders were age, exposure time, height, weight, and smoking. As a covariate, smoking was taken into the model as pack-years, but we also included it in the model as a factor, where it was classified. The statistical software used was SAS (SAS Institute, USA) and Egret (Egret, USA).

Results

SUBJECTS

The participation of exposed workers in this study was high, almost 100%, and for the controls the corresponding value was 72%.

The study groups were similar in height and weight (table 1). There was a five year difference between the mean age of the youngest (control) and the oldest (Cr^{+3}) groups. More than half of the workers in the Cr^{+3} and chromite groups were between the ages of 45 and 65 years. The chromite group contained more current smokers and fewer non-smokers than the other groups. Also, the chromite group smoked more and had smoked longer than the other groups.

Only three men had been previously exposed to chromium compounds in the metal industry. The groups were similar for former agricultural work and exposures to silica dust, welding fumes, asbestos, solvents, and textile dusts (data not shown). There were no differences between the number of reported earlier allergic or pulmonary diseases diagnosed by a physician. Allergic rhinitis and bronchial asthma were infrequent; no asthma was reported in the chromite or control group. The groups were similar with regard to medication for hypertension and cardiovascular diseases.

SYMPTOMS

The prevalence of most of the respiratory and other symptoms did not differ significantly in the comparison between the groups (table 2). The production of phlegm was more frequent in the Cr^{+3} and chromite groups than in the other groups. Work related cough or dyspnoea was significantly more frequent in the Cr^{+6} (P = 0·041) and the Cr^{+3} (P = 0·033) groups than in the control group.

No significant differences between the exposure groups and the controls were found in the odds ratio (OR) of the symptoms (table 3). In the logistic regression analysis age and smoking significantly explained the occurrence

*Table 4 Lung function measurements as a percentage of predicted values**

Lung function variable	Cr^{+6} group: Non-smokers (n = 41) mean (SD)	Cr^{+6} group: Smokers† (n = 67) mean (SD)	Cr^{+3} group: Non-smokers (n = 22) mean (SD)	Cr^{+3} group: Smokers† (n = 52) mean (SD)	Chromite group: Non-smokers (n = 6) mean (SD)	Chromite group: Smokers† (n = 30) mean (SD)	Control group: Non-smokers (n = 33) mean (SD)	Control group: Smokers† (n = 60) mean (SD)
FVC	96·4 (11·1)	93·5 (9·6)	93·8 (8·9)	90·7 (10·6) P < 0·05	93·6 (11·7)	86·8 (10·7) P < 0·01	93·5 (9·5)	95·2 (10·4)
FEV_1	95·5 (11·4)	91·8 (12·2)	93·8 (11·6)	89·1 (11·8)	92·1 (10·9)	83·1 (14·8) P < 0·01	94·6 (10·7)	92·7 (11·8)
FEV%	99·8 (6·6)	98·1 (7·9)	99·6 (6·9)	98·2 (7·1)	98·5 (6·3)	95·2 (10·3)	101·1 (5·9)	97·3 (6·9)
PEF	102·5 (11·9)	103·6 (13·2)	97·9 (18·0)	100·6 (14·1)	105·6 (7·9)	97·7 (12·9)	102·2 (11·6)	98·6 (14·4)
MEF_{50}	95·7 (21·3)	87·0 (26·1)	87·8 (30·8)	83·2 (25·4)	88·7 (18·9)	74·7 (28·3)	95·2 (22·2)	82·7 (22·7)
MEF_{25}	86·5 (26·1)	82·1 (32·7)	83·8 (31·4)	81·0 (27·5)	82·6 (26·1)	69·1 (28·8)	96·9 (33·4)	82·2 (30·3)
TLCO	102·3 (20·1)	97·2 (20·2)	109·3 (16·9)	90·4 (18·5)	85·5 (42·6)	82·1 (31·4) P < 0·05	102·4 (23·3)	95·6 (13·8)
$TLCO_{Hb}$	101·6 (20·1)	96·3 (19·4)	109·0 (15·5)	90·6 (18·4)	85·6 (42·6)	81·9 (31·2) P < 0·05	102·2 (22·8)	94·3 (14·0)
TLCO/VA	105·9 (23·6)	103·2 (20·7)	113·8 (17·1)	96·8 (20·6)	92·7 (48·3)	92·0 (35·9)	107·4 (26·2)	99·5 (14·6)
$TLCO/VA_{Hb}$	105·4 (23·6)	103·0 (20·8)	113·7 (15·6)	97·1 (20·6)	92·5 (47·8)	91·8 (35·9)	107·5 (26·2)	99·4 (15·1)

*Cr^{+6}, Cr^{+3}, and chromite groups *v* control group, Student's two tailed *t* test.
FVC = forced vital capacity; FEV_1 = forced expiratory volume in one second; FEV% = $FEV_1/FVC \times 100$; PEF = peak expiratory flow; MEF_{50} = maximum expiratory flow rate at 50% of the vital capacity; TLCO = diffusing capacity; $TLCO_{Hb}$ = diffusing capacity adjusted to haemoglobin; TLCO/VA = specific diffusing capacity; $TLCO/VA_{Hb}$ = specific diffusing capacity adjusted to haemoglobin.
†Smokers group includes both current and ex-smokers.

of most of the respiratory symptoms. Earlier allergic diseases were associated with the occurrence of shortness of breath (dyspnoea).

LUNG FUNCTION TESTS

In general the smokers showed lower lung function results. The smokers in the chromite group had significantly lower FVC, FEV_1, and diffusing capacity values than the smokers in the control group. The results of the lung function tests (as a percentage of the predicted values) of the Cr^{+6} and Cr^{+3} groups did not differ from the corresponding results of the controls, except for the FVC of the smokers in the Cr^{+3} group, for whom it was lower than for the smokers in the control group (table 4).

In the group comparison of the adjusted lung function results, modelling for interactions between study group, smoking, and exposure time did not show a significant difference for any of the lung function variables.

The same analysis of variance without interactions showed that the chromite group had lower FVC, FEV_1, and diffusing capacity values than the other groups, including the control group. The difference in these variables remained when smoking (pack-years) was included as a covariate in the model. The inclusion of smoking or pack-years or both significantly decreased the values of the same variables.

The percentages of the lung function test results that were below the predicted values[16] were similar among the exposed groups, except for the chromite group, for which all the diffusing capacity variables and the FVC, FEV_1, and MEF_{50} were significantly decreased (table 5).

Table 5 Prevalence of impaired‡ lung function test results

Lung function variable	Cr^{+6} group† (n = 109) %	Cr^{+3} group† (n = 76) %	Chromite group† (n = 36) %	Control group† (n = 95) %
FVC	10·0	16·0	33·3**	10·5
FEV_1	11·8	22·7	36·1**	13·7
FEV%	10·9	6·7	13·9	6·3
PEF	0·9*	5·3	5·6	6·3
MEF_{50}	12·7	17·3	27·8	13·7
MEF_{25}	13·6	10·7	27·8	10·5
TLCO	5·5	6·7	16·7**	2·1
$TLCO_{Hb}$	6·4	5·3	16·7**	2·1
TLCO/VA	4·6	4·0	13·9*	3·2
$TLCO/VA_{Hb}$	5·5	5·3	13·9*	3·2

*P < 0·05; **P < 0·01 (Cr^{+6}, Cr^{+3}, and chromite groups *v* the control group, Student's two tailed *t* test.)
†Smokers and non-smokers combined.
‡Value lower than the 5th percentile of distribution of the variable for adult non-smoking Finnish men.[16]
For abbreviations, see table 4.

RADIOGRAPHS

Table 6 shows the radiological findings. The number of positive findings increased with age in the Cr^{+6}, Cr^{+3}, and control groups.

Radiographic parenchymal abnormalities were found more often, but not significantly among the workers in the chromite group (table 7).

Changes in the parietal and visceral pleura were also more frequent (not significantly) among the workers exposed to chromite, who, however, had no bilateral plaques (table 8).

Table 6 Occurrence of small opacities (profusion ≥ 0/1) by age

Age group	Cr^{+6} group (n = 109) n (%)	Cr^{+3} group (n = 76) n (%)	Chromite group (n = 36) n (%)	Control group (n = 95) n (%)
29–44	5 (8·2)	1 (3·0)	7 (38·9)	6 (8·5)
45–54	7 (16·3)	8 (23·5)	2 (13·3)	4 (21·1)
55–65	1 (20·0)	5 (55·6)	3 (100·0)	2 (50·0)
Total	(12·0)	(18·4)	(33·3)	(12·8)

Table 7 Occurrence of small opacities

Profusion*	Cr^{+6} group (n = 109) n (%)	Cr^{+3} group (n = 76) n (%)	Chromite group (n = 36) n (%)	Control group (n = 95) n (%)
0/1	6 (5·5)	4 (5·2)	8 (22·2)	11 (11·7)
1/0	4 (3·7)	5 (6·6)	2 (5·5)	1 (1·1)
1/1	3 (2·8)	5 (6·6)	1 (2·8)	0 (0)
1/2	0 (0)	0 (0)	1 (2·8)	0 (0)
Total	13 (12·0)	14 (18·4)	12 (33·3)	12 (12·8)

*Classified according to the more severe finding when the interpretations of the two radiologists differed from each other.

URINARY CHROMIUM

The mean urinary concentration of chromium was 0·03 μmol/l for the 44 workers in the steel smelting shop (0·04 μmol/l in the previous study in 1987[12]). The maximum concentration was 0·08 μmol/l (0·34 μmol/l in 1987). The results of these control measurements indicate that the level of chromium exposure in 1993 was the same or slightly lower than in 1987.[12]

Table 8 Pleural plaques and changes in the visceral pleura

Variable	Cr^{+6} group (n = 109) n (%)	Cr^{+3} group (n = 76) n (%)	Chromite group (n = 36) n (%)	Control group (n = 95) n (%)
Bilateral pleural plaques	5 (4·6)	2 (2·6)	—	—
Unilateral pleural plaques	5 (4·6)	1 (1·3)	4 (11·1)	3 (3·2)
Changes in visceral pleura*	1 (0·9)	1 (1·3)	2 (5·6)	1 (1·1)

*Costophrenic angle obliteration or pleural adhesions or thickening of the visceral pleura.

Table 9 Description of chromium compounds[20]

Chromite	Chromium ore (Cr_2FeO_4), Cr^{+3}
Ferrochromium	Chromium alloy (Cr, C, Fe, N, Si), Cr^0
Chromates	Salts of chromic acid (H_2CrO_4), Cr^{+6}
Chromium	Metallic chromium (Cr), Cr^0

FORMER WORKERS

The questionnaire was returned by 37 former workers (70%). They reported that they had experienced the following symptoms during the years they were working for the company: frequent cough (16·2%), production of phlegm (16·2%), shortness of breath (13·5%), rhinitis (35·1%), eye irritation (8·1%), dermatitis (18·9%), and various symptoms (headache, vertigo, fatigue, etc, 16·2%).

None of the former workers reported that a disease had been a reason for leaving the company. At the time of the survey one person (2·7%) reported having chronic bronchitis and two people (5·4%) reported bronchial asthma. No other pulmonary diseases, allergic rhinitis, or cancer were reported.

Discussion

The respiratory effects of exposure to chromium compounds have been studied in various industries, but none of the studies cover the entire production chain of stainless steel. Because our objective was to study chronic effects, the requirement for a minimum duration of employment had to be kept high. Therefore the group sizes were small. This will decrease the statistical power of the study, as can be seen in the wide 95% confidence intervals (95% CIs) of the ORs for symptoms (table 3). On the other hand, the durations of exposure were long, the average ranging from 16 to 20 years in the different exposure groups. Lowering the durations of exposure would not have essentially increased the group sizes.

Most of the workers in this study had worked in the same production department during their entire employment at Outokumpu Oy. Therefore the exposures can be considered to be the same for all the people in any given department, occasional peaks being smoothed by time. Thus the scarcity of findings in this study is not due to the possibility that the pathological findings of a small, but highly exposed group had been diluted among a larger and less exposed group.

All the workers who had already left the company received a questionnaire, and 70% of them returned it. Half of the 70% had smoked while employed by the company. This group of former workers did not differ from the other groups for respiratory symptoms. Chronic bronchitis among the former workers was as rare as among the workers examined. Two former workers reported bronchial asthma, but none of the present workers did. None of the former workers reported a disease as a reason for leaving the company. Thus selection can be regarded as non-existent in this study.

The lung function tests could not be carried out in a randomised order for practical reasons. However, the lack of randomisation cannot be considered to have had any notable effect on the results because the tests were performed in a standardised manner.

Minor differences in the occurrence of cough and shortness of breath were seen when the Cr^{+6}, Cr^{+3}, and chromite groups were compared with the control group. However, in the logistic regression analysis no significant differences in the risk ratios were found between the groups.

The lung function results of our study could not be compared with the results from other studies because there are no such data available on stainless steel production.

As in this study epidemiological studies on respiratory functions very often indicate that the results of lung function tests of former smokers may surprisingly differ from the results of both non-smokers and current smokers. The reason for them having stopped smoking is often some effect on the respiratory system. Therefore in this study ex-smokers were included in the group of smokers in most of the analyses.

The results of the lung function tests in the groups exposed to Cr^{+6} or Cr^{+3} did not differ from those of the control group. On the contrary, the results on respiratory volumes and diffusing capacity in the chromite group were slightly worse than those of the control group. This difference was most evident for the smokers in this group, which had the largest proportion of smokers.

The airborne dust in the chromite mine has been shown to be one third chromite, one third talc, and one third chlorite serpentine. The talc and serpentine particles are primarily lamellar schists. Part of the chlorite serpentine particles can be classified as fibres. During the late 1960s and early 1970s, when the process was different and the technology less advanced, the dust concentrations in the ambient air at the mine were higher than the current concentrations.

Limited areas in the ore of the north eastern part of the mine and its waste rock contained crevices filled with chrysotile. During 1987–9 the mean concentration of fibres in personal samples was 0·14 fibres/cm³ for drillers and 0·11 fibres/cm³ for loaders at the open pit and 0·28 fibres/cm³ in the concentrating plant. According to scanning electron microscopy 42·5% of these fibres were chrysotile asbestos, and the rest comprised other particles—for example, vertical lamellar minerals.

The workers from the chromite mine were slightly older than the controls, and the proportion of smokers among them was greater. Therefore the lower lung function values of

these workers can be explained partly, but not totally, by the differences in age and smoking habits between the groups. It is also probable that there is a causal correlation between our findings and both the higher dust exposures during the first years of mine operation and the fibrous minerals in the waste rock of the mine.

Radiological parenchymal abnormalities and plaques classified according to ILO recommendations were not common in our study. Small opacities were more frequently observed in the radiographs of the workers exposed to chromite than in the radiographs of the other groups. This finding agrees with the idea that early exposure to fibrous materials is a factor contributing to the pulmonary effects.

An increased risk of lung cancer has been found among workers in the production of chromates and chromate pigments, as well as in chromium plating, although no conclusive data are available on lung cancer in ferrochromium production.[20 21] No cases of lung cancer were found in this study. However, risk of cancer could not be excluded because the follow up time was short and the exposed group young and small.

No cases of chromium asthma were found in this study. It was recently reported that mortality from non-malignant diseases of the respiratory system was not increased (standardised mortality ratio (SMR 0·88)) among production workers in stainless steel production.[21]

Conclusions

An average exposure time of 18 years in modern ferrochromium and stainless steel production and low exposure to dusts containing Cr^{+6} or Cr^{+3} does not lead to any respiratory changes detectable by lung function tests or radiography or to any increase in symptoms of respiratory diseases. The process chain under study is unique; however, the results are also applicable to other production facilities where the exposures to different chromium compounds are equally low.

The lung function test results were lower and the occurrence of radiological findings was more frequent among the workers from the chromium mine than among the controls. The difference was partly caused by differences in age and smoking habits, but evidently also partly by higher exposures more than two decades ago, when the mine operations were started, and by the fibrous components of the dust.

Because the follow up time was limited, we are planning a reinvestigation after five years, although there are no personal needs or expected benefits to individual workers. The main reason is to collect new information and to confirm the present results.

This study was conducted with financial support from the Finnish Work Environment Fund.

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