Review of the effect of pH on the integrity of *in vitro* and *in vivo* genotoxicity assays

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INTRODUCTION

The Precious Metals and Rhenium Consortium (PMRC) has requested advice on the appropriate testing programme for the REACH registration of a number of Platinum Group Substances. This involved a review of existing published and proprietary data and developing proposals for fulfilling the relevant Annex VII and VIII endpoints. A number of in-scope substances have low pH values and there is a need to understand the potential impact of this on the conduct of appropriate genotoxicity assays. As a result, an expert opinion has been sought on the role of pH in the conduct of genotoxicity studies and the reliability of any results obtained.

The objective of this commentary is therefore to advise the Precious Metals and Rhenium Consortium and its consultants on, among others:

- the potential confounding influence of extreme pH on the integrity and results from *in vitro* genotoxicity tests (particularly the mammalian cell mouse lymphoma and micronucleus assays) in relation to certain platinum group metals and salts;
- on (apparently) conflicting study results on Rh (III) compounds (rhodium trichloride/rhodium trinitrate); and
- to provide recommendations for the conduct of further genotoxicity tests in the context of REACH.

One key consideration is that the bacterial reversion assay (the “Ames test”) may not be considered appropriate for metal substances (HERAG factsheet No 5) due to the low sensitivity to metal genotoxicity of the bacterial strains used. Therefore the two *in vitro* assays that have been proposed for these substances are the mouse lymphoma assay (in place of the Annex VII requirement for the bacterial gene mutation assay) and the micronucleus test (Annex VIII), and these proposals are discussed.

SCOPE OF REVIEW

It is understood that extremes of pH or pH shifts may influence the reliability and conduct of *in vitro* genotoxicity assays, particularly in mammalian cells. Because of the potential influence of pH on study integrity, a recommendation has been made that the REACH registration programme will use the mouse lymphoma (MLA) and micronucleus (MN) assays to fill the (Annex VII & VIII) genotoxicity endpoints on the basis that the Ames test may be regarded as unreliable for the detection of metal genotoxicity (HERAG Factsheet 5). In order to perform the proposed tests on the identified substances, it is necessary to understand the conditions under which the tests can be conducted if the substance has a low pH, and the pH range in which the test results are considered to be reliable.

A number of substances have already been subjected to testing in the Ames test and in the proposed mammalian cell assays, and the potential influence of pH on the test results, requires consideration. The tests already performed are listed below together with the reported outcomes.

Tested *in vitro*:

- Rhodium trichloride "hydrate" (CASRN 20765-98-4) pH <2 (Merck)
  - Positive mouse lymphoma assay
  - Positive micronucleus assay
  - Positive Ames test
- Rhodium trinitrate (CASRN 10139-58-9) pH 1.3 (hydrate)
  - Negative Ames test
- Rhodium (III) acetate (CASRN 42204-14-8)
  - Positive Ames test
- Rhodium triiodide (CASRN 15492-38-3)
  - Positive Ames test
- Platinum dinitrate (CASRN 18496-40-7) pH 0.7 (hydrate)
  - Positive mouse lymphoma assay
  - Positive Ames test
- Hexachloroplatinic acid (CASRN 26023-84-7) pH 1 – 1.5
  - Positive mouse lymphoma assay
  - Positive Ames test

Tested *in vivo*:

- Rhodium trinitrate (CASRN 13465-43-5) pH 1.3
  - Negative mouse micronucleus test
- Rhodium trichloride "hydrate" (CASRN 20765-98-4) pH <2 (Merck)
  - Positive micronucleus test

The design of these studies, interpretation of the results (in particular in relation to pH) and the conclusions drawn, are discussed in detail in this review.

In addition the following substances are in scope and proposed for *in vitro* testing, and recommendations for which *in vitro* tests should be performed, in light of the proposed strategy and based on the known pH values for these substances, are discussed later in this review:

- Diammonium hexachlororuthenate (CASRN 18746-63-9) pH 2.6
- Dihydrogen tetrachloropalladate(2-) (CASRN 16970-55-1) pH 1.4
- Palladium dinitrate (CASRN 10102-05-3) pH 1.5

- Diamminedichloropalladium (CASRN 13782-33-7) pH 3.2
- Palladium (II) di(4-oxopent-2-en-2-oate) (CASRN 14024-61-4) pH 3.6
- Diammonium hexachloropalladate (CASRN 19168-23-1) pH not reported
- Dihydrogen hexahydroxyplatinate (CASRN 51850-20-5) pH 2.9
- Platinum dinitrate (CASRN 18496-40-70 (*in vitro* MN only*)* pH 0.7 (hydrate)
- Platinum, 1,3-diethenyl-1,1,3,3-tetramethyldisiloxane complexes/Karstedt concentrate pH not reported
- Tetrachloroauric acid (CASRN 16903-35-8) [using a suitable salt?] pH 1

In addition, (a suitable salt of) hexachloroplatinic acid (CASRN 26023-84-7, pH <1) is currently proposed for an *in vivo* micronucleus test based on the positive *in vitro* results, but if the latter are confounded by low pH values and therefore may not be considered valid, the appropriate programme for this substance needs to be discussed further. Hence an interpretation of the *in vitro* results is given and consequent recommendations made on the need (or not) to conduct the proposed *in vivo* MN test.

INITIAL COMMENTS ON pH EFFECTS

(a) Bacteria

Shifts to both low and high pH clearly affect the viability of bacteria. By contrast, extremes of pH (3-10) have been shown ***not*** to induce mutation in the Ames test either in the absence or presence of S9, using standard plate incorporation or pre-incubation methods (Tomlinson, 1980; Cipollaro *et al*, 1986). Also hydrochloric acid was not mutagenic in the Ames test (SIDS, 2005). Prolonged incubation at high (alkaline) pH has been shown to result in increased mutation predominantly in *Salmonella* strains TA97a, TA102 and TA104, but effects were only marginal in TA98 and TA100 (Musarrat and Ahmad, 1988). The data were interpreted to indicate effects at A-T rich regions of the DNA, but (at least in TA102 and TA104) could indicate oxidative damage. By including periods of liquid holding after the high pH exposures the authors were able to show the damage was repaired and revertant frequencies decreased.

(b) Mammalian cells

Effects of low (6.5-6.9) and high (8.4-8.8) pH on the stability of human chromosomes was reported as long ago as 1975 (Shimada and Ingalls, 1975). In this paper the authors reported changes in chromosome number and induction of endoreduplication, i.e. effects on cell division integrity. Acetic and hydrochloric acid both induced significant increases in chromosomal aberrations in CHO cells, but only in the presence of induced rat liver S9 (Brusick, 1986). In the mouse lymphoma *tk* mutation assay (MLA) there was no increase in mutant frequency in the absence of S9 when pH was lowered to 6.4, but a small (1.9-fold) increase was seen at pH 6.3 where survival was reduced to 24%. On the other hand, significant increases in mutant frequency were seen when pH was lowered in the presence of S9 (10-fold

increases at pH 6.0; Cifone *et al*, 1987). Increased pH had no effect on mutant frequency, even in the presence of S9, except at highly toxic (<10% survival) conditions.

The most comprehensive studies of the effects of pH on chromosomal aberrations (CA) have been performed by Morita and colleagues. Morita *et al* (1989) showed that high pH (up to 10.9) did not lead to increased CA in CHO-K1 cells in the absence of S9, and in the presence of S9 only a few CA were induced. On the other hand there were some increases in CA (mainly chromatid breaks) with low pH (5.5 or below) in the absence of S9, but more significant damage (both breaks and exchanges) was induced at pH 6.2 and below in the presence of S9. Further studies (Morita *et al*, 1992) showed that the clastogenic effects of low pH in the absence of S9 were more dramatic and seen at higher levels of survival in CHO and CHL cells than in V79 379A cells or human lymphocytes – in the latter there were no increases in CA until levels of toxicity well in excess of the normal target of 50% were reached.

One possible reason for the increased chromosomal damage in cells exposed, particularly, to low pH is diminished DNA repair. This was demonstrated by Yuan *et al* (2000) in mouse 3340 cells. Diminution of DNA repair may also explain the increased clastogenicity of some (but not all) known genotoxins at low pH (Morita *et al*, 1991), although this could equally be due to chemical instability.

In summary, there is a much greater likelihood of low pH inducing artefacts in mammalian cell tests than in bacteria, and effects seem to be more dramatic in the presence of S9, although effects in mammalian cells may be minimised by using human lymphocytes in whole blood cultures (high buffering capacity and anti-oxidant activity).

(c) *In vivo*

No reports have been found indicating that pH induces genotoxic effects *in vivo*.

REVIEW OF EXISTING DATA

Rhodium trichloride "hydrate" (CASRN 20765-98-4)

(a) Ames tests

Two separate Ames test studies have been performed on rhodium trichloride "hydrate". The first, performed at TNO in 1986, used 5 strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA1538) in the absence and presence of induced rat liver S9 in 2 independent experiments, using the plate incorporation method. The top concentration in each experiment was 250 µg/plate, which induced slight toxicity seen as a reduction in the background lawn of growth.

Control revertant numbers for TA1535 in the absence of S9 and TA98 in the presence of S9 were higher than one would normally expect, but control revertant numbers for all other strains were normal. Positive control chemicals induced significant increases in revertant numbers in all strains in the absence and presence of S9.

Although rhodium trichloride "hydrate" did not induce increases in revertant numbers of strains TA1535, TA1537 or TA1538, increases in TA98 and TA100 revertant numbers were seen to a similar extent both in the absence and presence of S9 in both experiments. Increases were dose related and

reached 5.5 to 6.1-fold in TA98, and 2.8 to 4.4-fold in TA100 at the top or second highest concentration. Such increases (>2-fold) are considered biologically significant for these strains. Responses in these strains would suggest a frameshift mutagenic activity.

The purity of the test material was not given in the report, although the rhodium content was stated as 37.6%. Unless the purity was very low, from the data (concentrations tested and responses seen) it is unlikely that the mutagenic effects were due to an impurity. No pH measures were included in the report. However, since there is no evidence that low pH causes mutation in the Ames test (see above) this is not a likely explanation.

It is concluded that rhodium trichloride "hydrate" was mutagenic in the Ames test both in the absence and presence of S9. The fact that reasonably high concentrations could be tested and clearly positive results obtained suggests that the Ames test can be used as an appropriate system for investigating the mutagenic activity of metals such as rhodium.

The second study, performed at Bioservice Laboratories in 2006, used a slightly different combination of *Salmonella* strains, namely TA98, TA100, TA1535, TA1537 and TA102, in line with current recommendations. Treatments were carried out in the absence and presence of induced rat liver S9 in 2 independent experiments, using the plate incorporation method. The rhodium trichloride "hydrate" was tested as a liquid, and therefore the top concentration in each experiment was 5 µL/plate, equivalent to around 5000 µg/plate for a substance with a density of 1. Toxicity (reduction in revertant numbers) was seen in most strains at treatments of 1 µL/plate or higher in different parts of the study.

Control revertant numbers for all strains were normal. Positive control chemicals induced significant increases in revertant numbers in all strains in the absence and presence of S9.

Although rhodium trichloride "hydrate" did not induce increases in revertant numbers of strains TA1535 and TA1537, increases in TA98, TA100 and TA102 revertant numbers were seen to a similar extent both in the absence and presence of S9 in both experiments. Peak responses in TA98 and TA100 were seen at concentrations between 0.316 and 1 µL/plate (i.e. comparable, on a µg/pate basis with the peak increases seen in the TNO study). Peak responses in TA102 were seen at the slightly higher concentration range of 2.5-5 µL/plate. Increases were dose related and reached 4.2 to 5.4-fold in TA98, 2.4 to 2.7-fold in TA100, and 6.0 to 8.2-fold in TA102. Such increases (>2-fold) are considered biologically significant for these strains. Responses in these strains would suggest some frameshift mutagenic activity, although oxidative DNA damage could also explain the pattern of results.

The purity of the test material was not given in the report, although the active component ($RhCl_3$) was stated as 15.95%. Unless the purity was very low, from the data (concentrations tested and responses seen) it is unlikely that the mutagenic effects were due to an impurity. No pH measures were included in the report. However, since there is no evidence that low pH causes mutation in the Ames test (see above) this is not a likely explanation.

It is concluded that rhodium trichloride "hydrate" was again mutagenic in the Ames test both in the absence and presence of S9. The results are consistent with those seen in first study, although the inclusion of TA102 revealed even stronger mutagenic responses. The fact that reasonably high concentrations could be tested and clearly positive results obtained suggests that the Ames test can be used as an appropriate system for investigating the mutagenic activity of metals such as rhodium.

(b) Mouse lymphoma *tk* mutation assay (MLA)

This study was performed at Bioservice Laboratories in 2006. L5178Y cells were treated with rhodium trichloride "hydrate" for 4 hrs in the absence and presence of S9 in a single experiment. After a 2-day expression period mutant colonies were selected using the microwell method. The top concentration in both parts of the study was 10mM, which is the maximum required according to test guidelines. This induced quite high levels of toxicity (>80% reduction in relative total growth, RTG) both in the absence and presence of S9.

The key data are summarised in the following table:

S9	Conc. (mM)	Mutant frequency (MF) x 10^{-6}	Toxicity (% reduction in RTG)
-	0	94.52	-
	2	555.70	20
	3	504.10	32
	5	910.36	59
	6	1629.94	72
	7	1589.33	78
	8	1071.23	78
	9	*2038.01*	*90*
	10	*2514.49*	*93*
+	0	105.86	-
	1	213.52	10
	3	359.63	26
	5	1033.32	60
	6	975.97	67
	7	987.08	68
	8	1246.00	72
	9	1380.86	85
	10	1637.00	86

Shaded boxes indicate mutant frequencies that exceed solvent control by more than the Global Evaluation Factor (GEF; Moore *et al*, 2006).

Figures in italics should be excluded from analysis because toxicity exceeded 90%.

It was reported that the pH of the medium was normal even up to the top (10 mM) concentration. Although the pH of rhodium trichloride "hydrate" in aqueous solution is reported to be <2.0, clearly the buffering capacity of the culture medium is able to control the pH around neutral until quite high concentrations are reached.

Control mutant frequencies (MF) were within expected ranges, and the positive control mutagens induced significant increases in MF both in the absence and presence of S9.

Although no statistical analysis was performed, biologically significant increases in MF were seen following rhodium trichloride "hydrate" treatment. The increases in MF occurred to similar extents both in the absence and presence of S9, and exceeded the MF in the controls by more than the Global Evaluation Factor (GEF) of 126 x 10^{-6} (for the microwell assay), which is considered by experts (Moore *et al*, 2006) to represent a biologically significant response. Some of the significant increases were seen at concentrations producing little or no toxicity, and therefore the responses cannot be explained as an artefact of high levels of cytotoxicity. At the mutagenic concentrations, although there were small (around 2-fold or less) increases in large colony MF, the increases in small colony MF were much greater (6 to 10-fold), indicating that the predominant mode of action probably involved chromosomal damage.

The MF responses seen with rhodium trichloride "hydrate" in the study are quite different from those described by Cifone *et al* (1987) for low pH conditions in the MLA, where:

- Increases in MF in the absence of S9 were small and did not exceed the GEF even at high (75%) toxicity
- Increases in MF in the presence of S9 that did exceed the GEF were only seen at pH levels that induced >70% toxicity.

Thus, given that no pH shifts were reported even at the top concentration, and the difference in the pattern of MF responses from that reported for low pH, it does not seem plausible that the mutagenicity of rhodium trichloride "hydrate" in this system is due to low pH.

It is concluded that rhodium trichloride "hydrate" was mutagenic in mouse lymphoma cells, both in the absence and presence of S9. The predominant mode of action seemed to involve induction of chromosomal damage. The increased mutagenic activity did not seem to be due to high cytotoxicity or pH shifts.

(c) *In vitro* micronucleus (MN) test

This study was performed at Bioservice Laboratories in 2006. V79 cells were treated with rhodium trichloride "hydrate" for 4 hrs in the absence and presence of S9 in a single experiment. After treatment medium was removed, cells were cultured for a further 20 hrs in the presence of cytochalasin B in order to generate binucleate cells from those that had divided. Cells were then harvested, gently swollen in mild hypotonic solution, and slides prepared and stained with acridine orange (a nucleic acid-specific stain). Two slides per test concentration were prepared and 1000 binucleate cells per slide (i.e. 2000 cells per concentration) were scored for presence of MN.

The top concentration scored was 10 mM in the absence of S9 and 7.5 mM in the presence of S9. Both of these top concentrations induced more than the 60% toxicity (reduction in cytokinesis block proliferation index, CBPI) required by current guidelines.

Control MN frequencies fell within the normal range, and were markedly increased by treatment with positive control chemicals, although the response to the positive control aneugen, colcemid, was not statistically significant.

The key results are summarised in the table below:

S9	Conc. (mM)	% MN	Toxicity (% reduction in CBPI)
-	0	1.1	-
	1.25	**5.25**	30
	3.75	**5.25**	61
	10	*5.5*	*80*
+	0	1.05	-
	1.25	**3.45**	23
	3.75	**5.35**	46
	7.5	*7.85*	*82*

Bold figures indicate statistically significant increases in MN.
Shaded boxes indicate MN frequencies that exceed the historical control range.

Figures in italics should be excluded from analysis because toxicity clearly exceeded 60%.

It can be seen that statistically significant increases in MN frequencies were induced at all 3 concentrations in both the absence and presence of S9. However, data at the top concentrations should really be excluded from evaluation since toxicity well in excess of the recommended 50-60% was induced. Thus at 2 concentrations in the absence of S9 and at 1 concentration in the presence of S9, MN frequencies increased to levels that were statistically significant and exceeded historical control ranges, thus indicating biological significance. In some cases the increases were associated with modest levels of toxicity, and are therefore not likely to be an artefact of high levels of cell killing. It should be noted that a no-effect concentration was not identified in this study.

The MN were not examined for presence of whole chromosomes (e.g. by using anti-kinetochore antibody), and so it not known whether a clastogenic or aneugenic mode of action was involved.

There is no mention of any pH changes in this study, so it not known whether it was observed or not. Therefore it is also not clear whether the medium used in this study (MEM) has the same capacity to buffer any pH changes as the medium used in the MLA (RPMI 1640). It may be relevant that during treatment in this MN test no serum was present (thereby reducing the buffering capacity) whereas during treatment in the MLA 3% horse serum was present.

It is concluded that rhodium trichloride "hydrate" did induce MN in V79 cells, both in the absence and presence of S9. The increased genotoxic activity did not seem to be due to high cytotoxicity, but due to lack of data the impact of pH shifts cannot be excluded.

(d) *In vivo* mouse peripheral blood micronucleus (MN) test

This study was performed at Bioservice Laboratories in 2007. Groups of 5 young healthy adult NMRI mice of both sexes were dosed intraperitoneally (ip) with rhodium trichloride "hydrate" (dissolved in NaCl) at 200, 500 and 1000 mg/kg. The top dose was determined to be the maximum tolerated dose

(MTD) in preliminary testing. Animals dosed with the top 2 doses showed signs of systemic toxicity, namely reduction of spontaneous activity, apathy, palpebral closure and rough fur. Negative (vehicle) and positive control (cyclophosphamide) groups were also included.

Blood samples were taken 44 hrs (all groups) and 68 hrs (top dose and vehicle control groups only) after dosing. Samples were prepared for flow cytometry, and reticulocytes (called polychromatic erythrocytes in the report, but strictly this term should be reserved for the immature erythrocytes whilst still in the bone marrow compartment) were identified by expression of the CD71 antigen. A total of 10,000 reticulocytes were scored for presence of micronuclei (MN-RET) from each animal.

MN-RET frequencies in control groups at both sampling times were normal, and were significantly increased after treatment with the positive control.

Based on the proportion of reticulocytes to total erythrocytes there was no evidence of toxicity induced by rhodium trichloride "hydrate" at any of the dose levels.

At 44 hrs, MN-RET frequencies increased in a dose-related fashion in both males and females treated with rhodium trichloride "hydrate". Frequencies reached 8-12x control levels in the top dose group, and actually exceeded the MN-RET frequencies in the positive control groups. Although no historical control data were presented in this report by the laboratory performing the study, historical control data for the same time period and same laboratory were included in the MN-RET study on rhodium trinitrate hydrate. The MN-RET frequencies in the rhodium trichloride "hydrate" treated groups clearly exceeded the historical control ranges presented in that report. They were therefore statistically and biologically significant. At 68 hrs, the MN-RET frequencies in the top dose group were 2-3x control levels – these were significantly different from controls by statistical analysis, but were much closer to the upper end of the historical control ranges presented in the rhodium trinitrate hydrate report. It should be noted that a no-effect dose was not identified in this study.

The pH of rhodium trichloride "hydrate" in aqueous medium has been reported as <2. Thus, the NaCl solution that was injected would have been at low pH. However, dilution of the dose in the peritoneal fluid would probably have tended to bring the pH towards neutral. In any case, there is no evidence from the published literature that low pH can lead to induction of MN *in vivo*.

It is concluded that rhodium trichloride "hydrate" did induce MN in the peripheral blood reticulocytes of mice at doses approaching the MTD. The effects were not associated with any obvious toxicity in the target tissue.

Rhodium trinitrate (CASRN 10139-58-9)

(a) Ames tests

Two separate Ames test studies have been performed on rhodium trinitrate hydrate. The first, performed at NOTOX in 2003, used 4 strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) plus 1 strain of *E. coli* (WP2*uvrA*) in the absence and presence of induced rat liver S9 in 2 independent experiments, using the plate incorporation method. This design complies with current requirements. The test substance was dissolved in water, and the top concentration in each

experiment was 3000 or 4000 μg/plate (dependent on strain), which did not produce any precipitate, but induced toxicity seen as a reduction in the background lawn of growth (formation of microcolonies) and reduced numbers of revertants.

Control revertant numbers for all strains were normal. Positive control chemicals induced significant increases in revertant numbers in all strains in the absence and presence of S9.

Rhodium trinitrate hydrate did not induce any notable or significant increases in revertant numbers of any of the strains, either in the absence or presence of S9 in either experiment.

The purity of the test material was not given in the report, but the rhodium content was determined by gravimetric analysis to be 35.7%, i.e. within the 35-37% specified range. No pH measures were included in the report.

It is concluded that rhodium trinitrate hydrate was not mutagenic in the Ames test either in the absence or presence of S9. The fact that high concentrations could be tested suggests that the Ames test can be used as an appropriate system for investigating the mutagenic activity of metals such as rhodium.

The second study, performed at Bioservice Laboratories in 2006, used a slightly different combination of *Salmonella* strains, namely TA98, TA100, TA1535, TA1537 and TA102, also in line with current recommendations. Treatments were carried out in the absence and presence of induced rat liver S9 in 2 independent experiments. The plate incorporation method was used for the first experiment and the pre-incubation method was used for the second experiment. The top concentration in each experiment was 5000 μg/plate (the maximum required), which produced some toxic effects in strain TA102 in the first experiment only. The test substance was also dissolved in water, but on this occasion precipitation was seen in all strains at the top concentration in the presence of S9 in experiment 1, and in all strains at the top 1 or 2 concentrations in the absence and presence of S9 in experiment 2.

Control revertant numbers for all strains were normal. Positive control chemicals induced significant increases in revertant numbers in all strains in the absence and presence of S9.

A 2.1-fold increase in TA1537 revertants was seen at the top concentration of rhodium trinitrate hydrate tested in the absence of S9 in experiment 2. However, this was not reproduced in the first experiment and does not reach a level (3-fold) considered biologically significant for this strain. There were no notable or significant increases in revertant numbers of any of the other strains treated with rhodium trinitrate hydrate, either in the absence or presence of S9 in either experiment.

The purity of the test material was not given in the report, but, unless the purity was very low, from the data (concentrations tested and responses seen) it is unlikely that the weak response in TA1537 was due to an impurity. No pH measures were included in the report.

It is concluded that rhodium trinitrate hydrate was again not mutagenic in the Ames test either in the absence or presence of S9, using plate incorporation and pre-incubation methodology up to maximum required and precipitating concentrations. The fact that high concentrations could be tested suggests that the Ames test can be used as an appropriate system for investigating the mutagenic activity of metals such as rhodium.

(b) *In vivo* mouse peripheral blood micronucleus (MN) test

This study was performed at Bioservice Laboratories in 2007. Groups of 5 young healthy adult NMRI mice of both sexes were dosed intraperitoneally (ip) with rhodium trinitrate hydrate (dissolved in NaCl) at 16, 40 and 80 mg/kg. The top dose was determined to be the maximum tolerated dose (MTD) in preliminary testing. Animals in the top 2 dose groups showed signs of systemic toxicity, namely reduction of spontaneous activity, palpebral closure, cramps and rough fur. Negative (vehicle) and positive control (cyclophosphamide) groups were also included.

Blood samples were taken 44 hrs (all groups) and 68 hrs (top dose and vehicle control groups only) after dosing. Samples were prepared for flow cytometry, and reticulocytes (called polychromatic erythrocytes in the report, but strictly this term should be reserved for the immature erythrocytes whilst still in the bone marrow compartment) were identified by expression of the CD71 antigen. A total of 10,000 reticulocytes were scored for presence of micronuclei (MN-RET) from each animal.

MN-RET frequencies in control groups at both sampling times were normal, and were significantly increased after treatment with the positive control.

Based on the proportion of reticulocytes to total erythrocytes there was no evidence of toxicity induced by rhodium trinitrate “hydrate” at any of the dose levels.

At 44 hrs, MN-RET frequencies were all slightly increased above vehicle control levels particularly in males treated with rhodium trinitrate hydrate. The maximum increase was in the mid-dose group (40 mg/kg), and was significantly different from vehicle controls, but only reached 1.6x control levels. None of the individual males in this group had MN frequencies that exceeded the maximum MN frequencies seen in historical vehicle controls. The increases were therefore considered not to be biologically significant. At 68 hrs, the MN-RET frequencies in the top dose group were identical to vehicle control levels.

The pH of rhodium trinitrate “hydrate” in aqueous medium has been reported as 1.3. Thus, the NaCl solution that was injected would have been at low pH. However, dilution of the dose in the peritoneal fluid would probably have tended to bring the pH towards neutral. In any case, there is no evidence from the published literature that low pH can lead to induction of MN *in vivo*.

It is concluded that rhodium trinitrate “hydrate” did not induce MN in the peripheral blood reticulocytes of mice at doses up to the MTD.

Rhodium (III) acetate (CASRN 42204-14-8)

(a) Ames test

This study was performed at TNO in 1986. Two independent experiments with 5 strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA1538) were performed using the

plate incorporation method in the absence and presence of induced rat liver S9. The test substance was dissolved in water and the top concentration was 10,000 µg/plate, which is higher than currently required but was commonly used at the time the study was performed. Some toxicity was seen at the top concentration. The design does not meet current requirements, in that strains detecting effects at A-T-rich sites, oxidative damage or cross-linking agents were not included, but the strains employed were in common use at the time.

Control revertant numbers for most strains were normal, however the TA1535 counts in the absence of S9 were unusually high, and the counts for TA98 in the absence of S9 were somewhat higher than would be expected. Positive control chemicals induced significant increases in revertant numbers in all strains in the absence and presence of S9.

No increases in revertant numbers for strains TA1535, TA1537 or TA1538 were seen either in the absence or presence of S9 following treatment with rhodium acetate. However, clear and reproducible increases in revertant numbers were seen, to similar extents in the absence and presence of S9, for strains TA98 and TA100. Peak increases were seen at quite high concentrations (between 3300 and 8000 µg/plate) reaching >3-fold in TA98 and >2-fold in TA100. The magnitude and reproducibility of the responses are considered biologically significant. However, mutagenic responses at such high concentrations may be due to impurities, and this cannot be excluded as only the rhodium content (39.54%) and not the purity was given.

It is concluded that rhodium (III) acetate is mutagenic in the Ames test both in the absence and presence of S9. The fact that high concentrations could be tested, and produced positive responses, suggests that the Ames test can be used as an appropriate system for investigating the mutagenic activity of metals such as rhodium.

Rhodium triiodide (CASRN 15492-38-3)

(a) Ames test

This study was performed at RTC in 2006. The first mutation experiment was performed with 4 strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) plus 1 strain of *E. Coli* (WP2*uvrA*) and used the plate incorporation method in the absence and presence of induced rat liver S9. The second mutation experiment used the pre-incubation method but did not include strains TA98 or TA100 as positive results had been obtained in the first experiment. The test substance was dissolved in DMSO and the top concentration was 5000 µg/plate, which produced some precipitation. The design complies with current requirements.

Control revertant numbers for all strains were normal, and positive control chemicals induced significant increases in revertant numbers in all strains in the absence and presence of S9.

There were no significant increases in revertant numbers for strains TA1535 or WP2*uvrA* in the first (plate incorporation) experiment. Increases of around 2-fold were seen for TA1537 revertants in the first experiment, but were not considered significant. In the second (pre-incubation) experiment, increases in TA1535 (2-fold), TA1537 (up to 3-fold) and WP2*uvrA* (<2-fold) were seen. These are quite weak responses and may not, in isolation, be considered biologically significant. However, in light of the larger increases in TA98 (2.7-fold) and TA100 (almost 6-fold) revertants seen in the first

experiment, these weaker increases in the other strains are probably a reflection of the mutagenic activity of rhodium triiodide. Peak increases were seen at the top 1 or 2 concentrations, but in TA100 biologically significant increases in revertant numbers of >2-fold were seen from the lowest concentration tested (313 μg/plate) upward, and so these are not likely to be due to impurities. The mutagenic responses in TA98 and TA100 were not confirmed in an independent experiment, but the dose responses and reproducibility of effect between treatments in the absence and presence of S9 indicate that are not likely to be due to chance.

It is concluded that rhodium triiodide is mutagenic in the Ames test both in the absence and presence of S9. The fact that high concentrations could be tested, and produced positive responses, suggests that the Ames test can be used as an appropriate system for investigating the mutagenic activity of metals such as rhodium.

Platinum dinitrate (CASRN 18496-40-7)

(a) Ames test

This study was conducted at SafePharm laboratories in 1999. Two independent experiments, with 4 strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) plus 1 strain of *E. coli* (WP2*uvrA*), were performed using the plate incorporation method in the absence and presence of induced rat liver S9. These experiments had used sterile water as the solvent. A confirmatory experiment was also performed in 3 of the strains using DMSO as solvent. This design complies with current requirements. The top concentration of platinum dinitrate (stated in the report as platinum IV nitrate) in each experiment was 5000 μg/plate, which did not induce any signs of toxicity, or form any precipitate. However, 5000 μg/plate is the maximum required for non-toxic, freely soluble materials, and therefore is a valid top concentration.

Control revertant numbers for all strains were normal. Positive control chemicals induced significant increases in revertant numbers in all strains in the absence and presence of S9.

Although platinum dinitrate did not induce increases in revertant numbers of strains TA1535 and TA1537, increases in TA98, TA100 and *E. coli* WP2*uvrA* revertant numbers were seen to a similar extent both in the absence and presence of S9 in all experiments, using both water and DMSO as solvents. Peak responses were usually seen at the top concentration. Increases were dose related but did not always reach the 2-fold level, particularly in TA100, considered biologically significant for these 3 strains. The increases were 2.3 to 5.6-fold in TA98, 1.3 to 3.2-fold in TA100, and 1.8 to 3.5-fold in *E. coli*. Although some increases were less than 2-fold, such reproducible dose-related increases are considered biologically significant. Responses in these strains would suggest both base substitution and frameshift mutagenic activity.

The purity of the test material was not given in the report. The relatively small (mainly 2 to 3-fold) increases in revertant numbers seen mainly at high concentrations could be due to a mutagenic impurity. No pH measures were included in the report. However, since there is no evidence that low pH causes mutation in the Ames test (see above) this is not a likely explanation.

It is concluded that platinum dinitrate was mutagenic in the Ames test both in the absence and presence of S9. The fact that high concentrations could be tested, and produced positive

responses, suggests that the Ames test can be used as an appropriate system for investigating the mutagenic activity of metals such as platinum.

(b) Mouse lymphoma *tk* mutation assay (MLA)

This study was performed at SafePharm laboratories in 2000. L5178Y cells were treated with platinum dinitrate (stated in the report as platinum IV nitrate) for 3 hrs in the absence and presence of S9 in 2 independent experiments. After a 2-day expression period mutant colonies were selected using the microwell method. The top concentration in experiment 1 was 2000 µg/ml, which induced 75-85% toxicity by reduction in relative total growth (RTG, the preferred measure of toxicity). This concentration proved too toxic in experiment 2 and the top concentration scored was 1750 µg/ml in the absence of S9 and 1500 µg/ml in the presence of S9, which induced 88 and 54% reductions in RTG respectively. Key results are summarised in the table below.

Expt.	S9	Conc. (µg/ml)	MF x 10^{-6}	Toxicity (% reduction in RTG)
1	-	0	57.06	-
		125	79.26	1
		250	68.24	-
		500	**131.51**	-
		1000	**212.60**	2
		1500	**356.57**	24
		2000	**645.89**	79
1	+	0	55.55	-
		125	69.60	-
		250	108.92	-
		500	**106.73**	-
		1000	**186.75**	-
		1500	**296.85**	-
		2000	**1056.60**	85
2	-	0	29.28	-
		250	28.43	1
		500	**76.30**	-
		1000	**67.82**	8
		1500	**196.13**	45
		1750	**427.93**	88
		2000	*619.35*	*99*
2	+	0	46.94	-
		250	67.31	22
		500	70.84	3
		1000	78.02	9
		1500	**191.38**	54
		1750	*297.86*	*97*
		2000	*197.90*	*100*

Bold figures indicate statistically significant increases in MF.

Shaded boxes indicate mutant frequencies that exceed solvent control by more than the Global Evaluation Factor (GEF; Moore *et al*, 2006).

Figures in italics were excluded from analysis because toxicity exceeded 90%.

It was noted that concentrations above 2500 µg/ml produced a visible change in colour of the pH indicator in the medium. Although the pH of platinum dinitrate in aqueous solution is reported to be 0.7, clearly the buffering capacity of the culture medium is able to control the pH around neutral until quite high concentrations are reached. Thus, platinum dinitrate was toxic at concentrations lower than those causing visible pH changes, and at the concentrations scored for mutants in this study there was no notable shift in pH in the cultures.

Control mutant frequencies (MF) were within expected ranges for most of the study, although in the absence of S9 in experiment 2 the control MF was unusually low. However, positive control mutagens induced significant increases in MF both in the absence and presence of S9.

It can be seen that increases in MF occurred to similar extents both in the absence and presence of S9, and exceeded the MF in the controls by more than the Global Evaluation Factor (GEF) of 126 x 10^{-6} (for the microwell assay), which is considered by experts (Moore *et al*, 2006) to represent a biologically significant response. Some of the significant increases were seen at concentrations producing little or no toxicity, and therefore the responses cannot be explained as an artefact of high levels of cytotoxicity. At the mutagenic concentrations, although the proportion of small colony mutants increased, the frequencies of both large and small colony mutants increased, indicating a mode of action involving induction of both gene mutations and chromosomal aberrations.

The MF responses seen with platinum dinitrate in the study are quite different from those described by Cifone *et al* (1987) for low pH conditions in the MLA, where:

- Increases in MF in the absence of S9 were small and did not exceed the GEF even at high (75%) toxicity
- Increases in MF in the presence of S9 that did exceed the GEF were only seen at pH levels that induced >70% toxicity.

Thus, given that pH shifts were only seen at concentrations much higher than those scored for mutants, and the difference in the pattern of MF responses from that reported for low pH, it does not seem plausible that the mutagenicity of platinum dinitrate in this system is due to low pH.

It is concluded that platinum dinitrate was mutagenic in mouse lymphoma cells, both in the absence and presence of S9. The mode of action seemed to involve induction of both gene mutations and chromosomal damage. The increased mutagenic activity did not seem to be due to high cytotoxicity or pH shifts.

Hexachloroplatinic acid (CASRN 26023-84-7)

(a) Ames tests

Two separate Ames test studies have been performed on hexachloroplatinic acid. The first, performed at NOTOX in 2002, used 4 strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) plus 1 strain of *E. coli* (WP2*uvrA*) in the absence and presence of induced rat liver S9 (5% mix) in 2 independent experiments, using the plate incorporation method. This design complies with current requirements. Hexachloroplatinic acid was dissolved in DMSO, and the top concentration in each experiment was 33 and 100 µg/plate in the absence and presence of S9 respectively. These

concentrations induced toxicity seen as a reduction in the background lawn of growth (formation of microcolonies).

Control revertant numbers for all strains were normal. Positive control chemicals induced significant increases in revertant numbers in all strains in the absence and presence of S9.

Hexachloroplatinic acid did not induce any notable or significant increases in revertant numbers of strains TA98, TA1535, TA1537 or *E. coli* WP2*uvrA*, either in the absence or presence of S9 in either experiment. Small (1.8 and 1.7-fold) increases in TA100 revertants were seen at the top concentration in the absence of S9 in both experiments. Whilst these may be considered not to achieve biological significance the 2-fold level was not reached, the reproducibility of effect indicates a weak mutagenic response. A 2.3-fold increase in TA100 revertants was seen at 33 µg/plate in the presence of S9 in the range-finding experiment, but this was not reproduced in either of the main mutation experiments even at higher concentrations.

The purity of the test material was not given, and no pH measures were included in the report. However, since there is no evidence that low pH causes mutation in the Ames test (see above) this is not likely to be a factor.

It is concluded that hexachloroplatinic acid was weakly mutagenic in the Ames test in strain TA100, with indications of activity both in the absence and presence of S9.

The second study was conducted at RTC laboratories in 2004. Two independent experiments, with 4 strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) plus 1 strain of *E. coli* (WP2*uvrA*), were performed in the absence and presence of induced rat liver S9 (10% mix). The first experiment used the plate incorporation method and the second experiment used the pre-incubation method. For reasons that are not clear, WP2*uvrA* was not tested in the pre-incubation method, so there are only data for this strain from a single experiment. Hexachloroplatinic acid was dissolved in water. Significant toxicity was seen at the higher concentrations investigated in preliminary tests, and particularly using the pre-incubation method, so the top concentration was limited to 10-80 µg/plate in the absence of S9, and 80-320 µg/plate in the presence of S9.

Control revertant numbers for all strains were normal. Positive control chemicals induced significant increases in revertant numbers in all strains in the absence and presence of S9.

Although hexachloroplatinic acid did not induce increases in revertant numbers of strains TA1535 and TA1537, increases in TA98, TA100 and *E. coli* WP2*uvrA* revertant numbers were seen in the absence but more strongly in the presence of S9 in both experiments. Increases were dose related but peak responses did not always reach the 2-fold level, particularly in TA100, considered biologically significant for these 3 strains. Apart from WP2*uvrA,* the increases in the absence of S9 were <2-fold. Increases in the presence of S9 were 2.5 to 4.3-fold in TA98, 1.95-fold in TA100, and 5.3-fold in *E. coli*. Although some increases were less than 2-fold, such reproducible dose-related increases are considered biologically significant, in this case particularly in the presence of S9. Responses in these strains would suggest both base substitution and frameshift mutagenic activity.

The purity of the test material was not given in the report, but the platinum content was given as 25.0%. Since positive mutagenic responses with hexachloroplatinic acid were seen at quite low concentrations, unless the purity was very low, it is unlikely that the mutagenic effects were due to an impurity. No pH measures were included in the report. However, since there is no evidence that low pH causes mutation in the Ames test (see above) this is not a likely explanation.

It should be noted that the concentrations used in this study were higher than in the previous NOTOX study. This is probably due to the use of water instead of DMSO as solvent. DMSO can form adducts with chloroplatinates that reduce their cytotoxic activity (Fischer *et al*, 2008), and therefore probably also reduce their mutagenic activity. It should also be noted that 10% S9 mix was used in this study whereas it was 5% in the NOTOX study.

It is concluded that hexachloroplatinic acid was more clearly mutagenic in this Ames test, particularly in the presence of S9. The differences in response from the previous (NOTOX) study are probably due to the use of water instead of DMSO as solvent, and perhaps the higher S9 concentration also modified the response. Although relatively low concentrations could be tested, the finding of reproducible positive responses suggests that the Ames test can be used as an appropriate system for investigating the mutagenic activity of metals such as platinum.

(b) Mouse lymphoma *tk* mutation assay (MLA)

This study was performed at NOTOX laboratories in 2003. L5178Y cells were treated with hexachloroplatinic acid, dissolved in ethanol, for 3 hrs in the absence and presence of S9 in a single experiment. After a 2-day expression period mutant colonies were selected using the microwell method. The top concentrations were 75 and 133 µg/ml in the absence and presence of S9 respectively, both of which induced >80% toxicity by reduction in survival. Relative total growth (RTG, the preferred measure of toxicity) was not measured in this study.

The key data are summarised in the table below.

It was noted in the solubility trials that at a concentration of 5000 µg/ml, the pH in the treatment medium was reduced to 2.58, compared to 7.42 in solvent control cultures. However, at 1000 µg/ml the pH was 6.99, so clearly the buffering capacity of the culture medium was able to control the pH around neutral until quite high concentrations were reached. Although the pH of the medium at the top concentrations of hexachloroplatinic acid scored for mutations (75 and 133 µg/ml) were not given, it can be extrapolated from the above that the pH at all the concentrations scored for mutants would have been >7. Hexachloroplatinic acid was therefore toxic at concentrations lower than those causing measurable pH changes.

Control mutant frequencies (MF) in this study were low (12-40 x 10^{-6}), compared with a minimum of 50 x 10^{-6} recommended by Moore *et al* (2003) for the microwell method. Nonetheless, positive control mutagens induced significant increases in MF both in the absence and presence of S9.

No statistical analysis was performed. The criteria for a positive response given in the report were induction of at least a 3-fold increase in MF accompanied by a dose-response. Different criteria are now used for evaluation of MLA data (Moore *et al*, 2006), although the Global Evaluation Factor

(GEF) signifying a biologically significant mutagenic response (increase in MF of 126 x 10^{-6}) would strictly only apply to experiments where control MF greater than 50 x 10^{-6} was achieved.

S9	Conc. (µg/ml)	MF x 10^{-6}	Toxicity (% reduction in survival)
-	0	13	-
	1	36	-
	3	33	-
	10	47	8
	18	91	46
	24	160	59
	32	166	53
	56	325	88
	75	*371*	*95*
+	0	31.5	-
	10	40	-
	18	43	-
	32	34	2
	42	43	-
	56	73	8
	75	86	-
	100	216	54
	133	143	83

Shaded boxes indicate mutant frequencies that exceed solvent control by more than the Global Evaluation Factor (GEF; Moore *et al*, 2006), even though this is a conservative approach with such low control MF.

Figures in italics were excluded from analysis because toxicity exceeded 90%.

Even though with this data set application of the GEF would be a conservative approach, it can be seen that biologically significant increases in MF occurred to similar extents both in the absence and presence of S9. Some of the significant increases were seen at concentrations producing only modest levels of toxicity, and although cell survival is not the recommended measure of cytotoxicity, it is unlikely that the responses could be due to an artefact of high levels of cytotoxicity. At the mutagenic concentrations, although the frequency of small colony mutants increased, the frequency of large colony mutants also clearly increased (up to nearly 10-fold), indicating a mode of action involving induction of both gene mutations and chromosomal aberrations.

The MF responses seen with hexachloroplatinic acid in the study are quite different from those described by Cifone *et al* (1987) for low pH conditions in the MLA, where:

- Increases in MF in the absence of S9 were small and did not exceed the GEF even at high (75%) toxicity
- Increases in MF in the presence of S9 that did exceed the GEF were only seen at pH levels that induced >70% toxicity.

Thus, given that the pH measurements made in this study indicated normal (around pH7) levels were achieved at the concentrations scored for mutants, and given the difference in the pattern of MF responses from that reported for low pH, it does not seem plausible that the mutagenicity of hexachloroplatinic acid in this system is due to low pH.

It is concluded that hexachloroplatinic acid was mutagenic in mouse lymphoma cells, both in the absence and presence of S9. The mode of action seemed to involve induction of both gene mutations and chromosomal damage. The increased mutagenic activity did not seem to be due to high cytotoxicity or pH shifts.

DISCUSSION OF EXISTING RESULTS

The finding of clear positive results in the Ames test with 5 of the 6 metal salts tested is perhaps surprising given that the Ames test is often considered insensitive to mutagenic/carcinogenic metals. Metals are often toxic to bacteria, and, even if they have mutagenic properties, can induce large DNA deletions which result in dead rather than revertant bacteria. Thus, the Ames test can give "false negative" results with such compounds. In the tests described above high concentrations of metal salts were tolerated for 5 of the substances, and even with hexachloroplatinic acid, which was the most toxic of the six, the lower concentrations tolerated produced mutagenic responses. There is also some consistency in the types of mutagenic responses observed, namely positive responses seen most clearly in strains TA98 or TA100, with usually weaker effects seen in *E. coli* WP2*uvrA*. Also, mutagenic responses were usually similar both in the absence and presence of S9, with only platinum dinitrate showing clearer mutagenic responses in the presence of S9.

The different Ames test results with rhodium trichloride and trinitrate are curious. Both were able to be tested to high concentrations, and if the mutagenic activity was due to rhodium ions, then similar results would have been expected. The fact that rhodium trinitrate produced precipitate (at least in 1 of the 2 studies), whereas rhodium trichloride did not, may provide an explanation. Perhaps it was not possible for sufficient rhodium molecules to enter the bacteria and attack the DNA in the trinitrate treatments, due to the presence of precipitate.

The mutagenic activity of rhodium trichloride in the Ames test is clearly reflected in the other 3 tests performed on this substance. Clear positive responses were obtained in the MLA, again in the absence and presence of S9, which could not be attributed to pH shifts or high cytotoxicity. Clear positive responses were also seen in the absence and presence of S9 in the *in vitro* MN test, and these could also not be attributed to high cytotoxicity, but the impact of pH is not known because it was not recorded in this study. Thus, rhodium trichloride appears to exhibit both gene mutation and chromosome damaging activity *in vitro*.

Rhodium trichloride also induced MN in mouse blood reticulocytes *in vivo*. The increases in MN frequency were large (8-12-fold), clearly exceeding the historical control range (obtained from the rhodium trinitrate study report), and therefore not likely to be due to chance. There are no published data to indicate that pH shifts can lead to artefactual genotoxic effects *in vivo*. Since analysis of rhodium in the blood plasma was not performed, it is not possible to correlate the *in vivo* genotoxic effects with concentrations found to be genotoxic *in vitro*.

The differences between the *in vivo* MN results for rhodium trichloride and trinitrate are also curious – the trichloride was clearly genotoxic whereas the trinitrate induced only very weak effects, mainly in

males at a mid-dose, that were considered not to be biologically relevant. Again if rhodium itself possesses genotoxic properties, then we may have expected rhodium trinitrate to also induce MN *in vivo*. There are several possible explanations for this discrepancy. Firstly, the doses of rhodium trinitrate that were tolerated were much (>10-fold) lower than the doses of rhodium trichloride. Secondly, the lower administered doses could have led to much lower systemic exposures, and as there was no measurement of rhodium in the target tissue (blood), this cannot be verified. Thirdly, because rhodium trinitrate was seen to precipitate in the Ames test, it may have precipitated in the peritoneal cavity after ip dosing, thus further reducing the systemic exposures.

The large number of positive results obtained *in vitro* and *in vivo* for rhodium compounds suggests that compounds that can produce trivalent rhodium ions in solution possess mutagenic properties. This is consistent with the findings of the Dutch Expert Committee on Occupational Standards (DECOS, 2002). This report includes data on other rhodium (III) compounds not evaluated here (because reports were not available) showing additional positive mutagenic responses. The report also notes that a study of rhodium chloride with isolated calf thymus DNA suggested binding of rhodium (III) to the phosphates and bases of DNA. Thus, the negative genotoxicity results with rhodium trinitrate seem to be the exception rather than the rule for soluble rhodium (III) compounds, and perhaps the explanation lies in the lower solubility of the trinitrate.

Both of the platinum compounds were mutagenic in the Ames test, although the responses were not as marked as with rhodium trichloride. One of the two Ames tests with hexachloroplatinic acid was negative, but that was when using DMSO as solvent, which is known to form adducts with chloroplatinates and thus reduce their biological activity. However, both salts gave strongly positive responses in the MLA both in the absence and presence of S9 that could not be attributed to pH shifts or high cytotoxicity. A review by the US EPA (2009) of the mutagenicity of 16 platinum compounds concluded that soluble platinum compounds that have a propensity to form charged complexes and have labile leaving groups (e.g. platinum chloride) appear to have mutagenic activity. However, there are limited data on several soluble platinum compounds and so it is not clear whether this conclusion is widely applicable. With several compounds the data are confusing, but this may be because of the effect of the solvent used, with DMSO producing reactive products that may make them less able to manifest their mutagenic properties than if water was used (Fischer *et al*, 2008). There are also suggestions that the structure of platinum complexes in DMSO is different from that in distilled water. For example, displacement of the chloride ligand with DMSO can occur when they are dissolved in DMSO, giving ionic species that react less strongly with bacteria. It is also possible that the susceptibility of the bacteria to mutagenic chemicals is changed in the presence of a large quantity of a solvent such as DMSO.

This series of metal salts appears to show a fairly consistent pattern of genotoxic responses, inducing both gene mutations and chromosomal damage *in vitro*. If sufficiently high exposures can be achieved, it seems they can also induce genotoxic effects *in vivo*. The apparently discrepant results with rhodium trinitrate may be explained by differences in exposure – precipitation probably affecting cellular or tissue uptake both *in vitro* and *in vivo*, and the lower *in vivo* doses also limiting systemic exposure.

APPROACHES TO FUTURE TESTING

It has been argued in HERAG Factsheet 5 that the Ames test may not be appropriate for testing metals for genotoxic potential. The main reason for the lack of sensitivity of the Ames test to mutagenic metals is that many are believed to induce large deletions of the DNA, and if the target (*his* or *tryp*) gene is deleted by action of the chemical, then dead rather than reverted bacteria result. Obviously such a scenario leads potentially to "false negative" results, i.e. true genotoxins not being detected because they have induced lethal rather than viable changes to DNA. It has been proposed that a mouse lymphoma *tk* mutation assay (MLA) should be conducted in place of the Ames test for evaluation of the mutagenic effects of metals. This would be appropriate for compounds to which the Ames test would not be sensitive. However, 5 of the 6 salts that were tested in the Ames test (rhodium trichloride "hydrate", rhodium (III) acetate, rhodium triiodide, platinum dinitrate and hexachloroplatinic acid) gave clear positive results, and in most cases high concentrations could be tested. The discrepant results with rhodium trinitrate may be explained by precipitation. Thus, based on these results, the Ames test does not appear to be susceptible to "false negative" results with metal salts of this series. Furthermore, there is no evidence that low pH produces artefacts in the Ames test. **Therefore, it can be argued that the Ames test is an appropriately sensitive test for metals of the rhodium/platinum series, and does not need to be replaced by the MLA.** However, care should be taken in the choice of solvents in particular avoiding use of DMSO with chloroplatinates, and on the concentration of S9.

The rationale for including the *in vitro* micronucleus (MN) test for detecting chromosomal damage is sound. This assay has the advantage that it can detect aneugenic (chromosome loss) events as well as structural damage to chromosomes. A recent publication (Kirkland *et al*, 2011) has demonstrated that a combination of the Ames and *in vitro* MN tests is sufficient to detect rodent carcinogens and *in vivo* genotoxins. This strategy has now been adopted by the UK Committee on Mutagenicity and EFSA in their latest recommendations.

The *in vitro* MN test can be conducted in human lymphocytes, and, as discussed earlier, the buffering capacity of human blood cultures seems to be more robust at dealing with low pH effects than cultures of cloned cell lines. Moreover, if there is a positive MN response with a test substance, pan-centromeric probing can be used with diploid human cells to determine if the mode of action is aneuploidy. **Thus, it is recommended that the Ames test is supplemented with an *in vitro* MN test in human lymphocytes.**

It should be noted that culture media for mammalian cells has a high buffering capacity, and contains a pH indicator. Thus shifts in pH due to a test chemical may well be limited, and any that are not adequately buffered should be obvious as the pH indicator changes colour (from red at neutral pH to yellow at low pH or purple at high pH). If unbuffered pH changes are seen, then experiments can be conducted with and without neutralisation (addition of NaOH in the case of low pH shifts, or addition of HCl in the case of high pH shifts). **Thus, even with *in vitro* MN studies in human lymphocytes, care should be taken to monitor changes in the colour of pH indicators in the medium, and additional experiments at neutral pH considered.**

This strategy (Ames + *in vitro* MN test in human lymphocytes) should be satisfactory for the list of compounds yet to be tested, even though they are predicted to give low pH values in aqueous medium, for the reasons given above. However, it is noted that some palladium compounds are included in the list, and as they have not been included in the existing assessment it is not known whether they will fit

the pattern seen for rhodium and platinum compounds. Thus, for the palladium compounds care should be taken to see if (a) the compounds are very toxic to the Ames bacteria, in which case a test for gene mutations in mammalian cells (see below) might be more appropriate, and (b) whether pH indicator changes occur in mammalian cell culture medium at concentrations selected for scoring, in which case tests under neutralised conditions may be needed.

A test for mutations in mammalian cells would be required under REACH if the Ames and *in vitro* micronucleus tests gave negative results. This requirement seems excessive in light of the recent recommendations of UK COM and EFSA – negative results in this 2-test battery is considered sufficient to exclude genotoxic potential. It is not clear at this time whether REACH recommendations will change to come into line with the latest scientific thinking as propounded by UK COM and EFSA, but in the meantime it may be necessary to conduct this additional mammalian cell gene mutation test. It is recommended to avoid the mouse lymphoma *tk* mutation assay (MLA), either as an alternative to the Ames test or at this stage of follow-up testing, since low pH effects have been reported to lead to increased mutation in this system as discussed earlier. Although there is no evidence that low pH contributed to the positive MLA results with rhodium trichloride, platinum dinitrate or hexachloroplatinic acid, there is a potential for confounding pH effects in these cells, particularly as they do not have functional p53, and may therefore be more susceptible to irrelevant genotoxic effects than p53-competent cells. This susceptibility to low pH effects is most likely associated with chromosomal damage, and since this will already have been measured in the *in vitro* micronucleus test, a different genetic locus could be studied (HPRT mutation to detect true gene mutations and not large-scale chromosomal damage) that is believed to be less susceptible to pH effects. Due to problems of statistical insensitivity in measuring HPRT mutations in monolayer Chinese hamster cells (CHO and V79 cells have been commonly used, but cell numbers are restricted due to a phenomenon called “metabolic co-operation”) it is preferable to perform HPRT mutation studies in mouse lymphoma L5178Y cells. However, although the risk of pH effects at this locus would be minimised, the p53 deficiency may still lead to a susceptibility to “false positive” results for other reasons. It may therefore be preferable to measure *tk* mutations in p53-competent human lymphoblastoid TK6 cells.

As discussed earlier, there is no evidence that pH shifts can interfere with *in vivo* tests such as the bone marrow or blood reticulocyte MN tests, and therefore this test system is considered appropriate to follow up on positive *in vitro* results. The measurement of MN in reticulocytes (rather than bone marrow) has been performed in the *in vivo* studies performed so far, and should therefore be the choice of test for the future to ensure similar statistical power and sensitivity are achieved. Of course, the pH of the administered dosing solution may affect the ability of the test substance to be absorbed, and possible precipitation (particularly if the ip route is used) should be considered. **However, the stomach maintains a low pH, and it is therefore suggested that the confounding effects of a low pH dosing solution could be minimised by using the oral route of administration. The oral route is, in any case, considered to be more physiologically relevant than the ip route.**

When an *in vivo* study is used to follow up on positive results *in vitro*, and gives a negative result, it is important to be able to demonstrate that the target tissue (bone marrow or blood in the case of an MN test) was exposed. Negative results with no evidence of target tissue exposure are considered to be of very limited value. Occasionally toxic effects will be seen directly in the target tissue (e.g. reduction in % PCE in the bone marrow or % reticulocytes in the blood), and thus exposure can be verified. However,

this only occurs in a small percentage (perhaps around 5%) of studies. **Thus, it is recommended that satellite animals are included and that blood samples are taken for toxicokinetic analysis of test substance in plasma.**

It should be noted that whilst the *in vivo* MN test is an appropriate follow-up to substances producing positive results in an *in vitro* MN test, it may not be appropriate for a substance that is only positive in the Ames test. Such a substance (i.e. producing only gene mutations) may not be satisfactorily assessed *in vivo* if only MN are measured. In such a case performing a transgenic mutation or Comet assay would be considered more relevant. These tests have the advantage that multiple tissues can be sampled. **Given that different choices are available for *in vivo* follow-up to positive *in vitro* results, and that the endpoints and tissues to be selected will be determined by the nature of the positive *in vitro* results, it is recommended that these choices are discussed before decisions are made.**

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