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March 19, 2012

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Attn: Section 8(e) Coordinator
U.S. Environmental Protection Agency
1201 Constitution Avenue, NW
Washington, DC 20004-3302



Re: TSCA Section 8(e) Submission for Vanadium Pentoxide (CASRN 1314-62-1)

Dear Sir or Madam:

On behalf of its members, the Vanadium Producers & Reclaimers Association (VPRA) submits to the U.S. Environmental Protection Agency (EPA) the following information under section 8(e) of the Toxic Substances Control Act (TSCA), 15 U.S.C. § 2607(e) for vanadium pentoxide (V₂O₅), Chemical Abstracts Service Registry Number (CASRN) 1314-62-1. These results concern preliminary testing to investigate potential mechanisms of inhibition of DNA repair. Comments were made by the study monitor, Dr. Douglas McGregor on February 21, 2012 regarding the results and subsequently we obtained a written report of the very preliminary findings. This is attached.

This notice is being filed within 30 days of the initial remarks. The findings being reported are based on a preliminary study involving the *in vitro* incubation of the test compound with mouse and rat lung tissue extracts from one mouse and one rat to investigate DNA repair efficacy using certain multiplexed DNA repair functional assays. The results are related to range finding activities associated with the preparation for more detailed studies. Additional results will be evaluated for submission under section 8(e) as they become available.

* * *

If you have any questions regarding this submission, please do not hesitate to contact me.

Sincerely yours,

John Hilbert, President
The Vanadium Producers & Reclaimers
Association



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Attachment

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ATI study- Subcontract 2012-502

Report 5: Preliminary experiments with V₂O₅ and Cadmium
February 29, 2012

Summary

Vanadium pentoxide is a suspected carcinogen. The aim of the present study is to gain insights in the possible interference by vanadium pentoxide (V₂O₅) in different DNA repair systems. For this purpose the consequences of the *in vitro* incubation of the compounds with mice and rat lung tissue extracts on DNA repair efficacy were studied using highly informative multiplexed DNA repair functional assays. After optimisation of the experimental conditions (protein concentration, repair reaction time) the effect of serial concentrations of V₂O₅ on DNA repair activities was investigated. The V₂O₅ concentrations used were relevant for comparison with concentrations in lung having toxicological consequences.

Although up to now only preliminary experiments aimed at determining the optimal incubation time between the compounds and the extracts have been conducted, it seemed that certain glycosylases that excise some DNA lesions (notably 8oxoG and hypoxanthine) are inhibited by V₂O₅. In addition, this compound appears to inhibit other excision activities and polymerases at higher *in vitro* concentrations that are nevertheless encountered *in vivo*. These experiments will be repeated using different extracts preparations and a better titration of the inhibitory properties of the compound will be performed to confirm the V₂O₅ action. Parallel experiments with CdCl₂ used as a positive control will also be done at optimized concentrations.

Introduction

The objective of this study is to examine whether vanadium pentoxide interferes with the activity of DNA repair enzymes that could be involved in base excision repair (BER) or, even, nucleotide excision repair (NER), perhaps as a consequence of interaction with DNA of vanadium ions rather than reactive oxygen species (ROS) generated from the pentoxide. This information is important for the formulation of a mode of (carcinogenic) action hypothesis and may be crucial for regulatory decisions.

In a 2009 study conducted at Harlan Laboratories, Switzerland, vanadium pentoxide was administered by nose-only inhalation to female B6C3F1 mice for a period of 16 days (6 h/day) at target concentrations of 0, 0.25, 1.0 and 4.0 mg/m³ in air (Schuler et al., 2011). The exposure levels used in this experiment were ones that, when applied over many months, resulted in a high incidence of lung tumours in the same mouse strain in the published US National Toxicology Program (NTP) whole-body exposure study of vanadium pentoxide (NTP Technical Report 504). A number of investigations were conducted on these mice, including a study of DNA damage in pulmonary epithelial and bronchio-alveolar lavage

(BAL) lavage cells as detectable by the single-cell gel electrophoresis (or "comet") assay and a study of a number of specific DNA lesions by mass spectrometry (MS). This type of lesion-specific study is necessary when dealing with small adducts created by oxygen species because the radioactivity measurements that are available when studying organic adducts are not applicable. The comet assay for DNA strand breaks and alkali-sensitive sites failed to demonstrate such damage in either lung or BAL cells. Also, of the 9 lesions of DNA investigated, only two were of sufficient concentration to appear above the level of detection (LOD) and of these two, only 8-oxo-7,8-dihydroguanine (8-oxodGuo) concentration in DNA of mouse lung was increased with increasing exposure to vanadium pentoxide. 8-OxodGuo is the most abundant oxidative lesion to be found in DNA and, consequently, the one most commonly reported, although many laboratories performing such studies have not demonstrated that their measurements follow the optimised methods required to eliminate or minimise the generation of this lesion as an artefact. Although surprising, this would seem to confirm that the only oxidative lesion that was increased was 8-oxodGuo. However, it is likely hydroxyl radicals ($\bullet\text{OH}$) are produced from vanadium pentoxide and $\bullet\text{OH}$ would not be predicted to produce such specific damage. Consequently, it appears that some difficult questions remain regarding an interpretation of the inhalation study.

Another possible reason for the higher 8-oxodGuo measurement has to be considered. The specific increase in the 8-oxoGuo lesion could have occurred not as a result of induction, but of interference with DNA base excision repair (BER) processes. 8-oxodGuo lesions are not only common but are produced as a result of ROS generated by endogenous biochemical reactions that are part of normal metabolism quite independently of exposure to vanadium pentoxide or any other exogenous chemical introduced to the target cells. Thus, if an agent (e.g., vanadium pentoxide) should inhibit the repair of such lesions then they would be more prevalent in the presence of the repair-inhibiting chemical than in its absence, giving the impression of induction.

Materials and Methods

Vanadium pentoxide batch number H110435, purity 100.33%, a yellow-orange solid was supplied by Stratcor Inc., 4343 Malvern Road, Hot Springs, Arkansas 71901, U.S.A. to CEA, Grenoble, France, where it was stored at room temperature (range of 20 ± 5 °C), under dry conditions. The transfer was according to GLP shipping, handling and record keeping procedures. This sample had been analysed for impurities (Appendix I: Certificate of Analysis).

I. Ethics - Animals

The present research protocol (entitled « Effet in vitro du vanadium pentoxide sur les activités de réparation de l'ADN d'extraits tissulaires de poumons chez le rat et la souris ») was approved (approval number 83_iRTSV-INAC-SS-01) by the local ethical committee of the Grenoble University (No. 12, Cometh-Grenoble) which is registered with the National Committee of Reflection on Ethical Animal Experiments of the Ministry of Research (France). The same protocol (08044004.11 entitled "In vitro effect of Vanadium Pentoxide on the repairing of DNA from Rat and mouse Lung Tissue Extracts) was approved by the USAMRMC Animal Care and Use Review Office (ACURO) award number W81XWH-09-2-0066.

The principal investigator of the study (Dr Sylvie Sauvaigo) has her national certificate allowing her to perform experimentations on rats and mice (N°38 11 27).

The Animal Care and Use policy that is applied in the Laboratory Animal Facility of the CEA's Research site (CEA Grenoble, France) is similar to and in accordance to the National Research Council's guidelines (USA).

Four female B6C3F1/OlaHsd Mice, aged 8 weeks from Harlan and 4 female Fischer 2F Rats, aged 8 weeks from Charles River (Harlan F344 rats were contaminated by a bacteria not acceptable within the Grenoble animal facility) were used for the experiments.

II. Assays used to measure the DNA Repair activities contained in rodent lung extracts

- Oligo Biochip: parallel measurement of Glycosylase/Lyase activities contained in rodent extracts.

Lesion- Paired base	Putative repair activity measured
EthenoA-T (EtheA)	MPG (AAG)
Hypoxanthine-T (Hx)	MPG (AAG)
Uracil-G (U)	SMUG, UNG ?
THF-A (AP site)	AP endonuclease
8oxoG-C	OGG1
A-8oxoG	MYH
Thymine Glycol-A (Tg)	NTH1
Uracil-A	No activity detected in rodents with our assay

- Plasmid Biochip (Basic version): parallel measurement of Excision/Synthesis Repair activities contained in rodent extracts: excision by Nucleotide Excision Repair (NER) enzymes and synthesis by polymerase delta/eta or by Bases Excision repair (BER) and synthesis by polymerase beta/delta and/or proteins from Recombination for Intra Strand Cross Links incision (ICLR) and synthesis by polymerases delta/eta.

Lesion	Putative repair activity measured
Photoproducts (CPD-64)	NER (recognition DDB1,2)
8oxoG	BER (OGG1)
Mix of alkylated bases (AlkB)	BER and NER
Cisplatin adducts (CisP)	NER (recognition HMGB4) + ICLR
Abasic sites (AaS)	APE1
Glycol (Cytosine and Thymine Glycol)	BER (NTH1)
Psoralen adducts (Pso)	NER + ICLR

Plasmid Biochip (Klenow version): parallel measurement of Excision activities of BER, NER and possibly ICLR contained in rodent extracts. The resulting breaks are subsequently labelled by the Klenow fragment of polymerase I.

III. Exploratory studies

1. Choice of euthanasia method

Effect of euthanasia method on repair activities: comparison of Dolethal intraperitoneal injection (1 mouse) with cervical dislocation (1 mouse) → no noticeable effect on repair activities (tested for the 3 assays in the conditions described below).

Conclusion: intra peritoneal injection of Dolethal was used for all animals' euthanasia.

2. Preparation of lung extracts

Before removal of the lungs, the hearts of the animals were perfused with PBS- 2 mM EDTA (about 8 ml) until lung blanching.

The lungs were removed and cut in small pieces (2-3 mm³) and transferred in Hanks medium until processing. They were subsequently rinsed in 1 ml PBS and centrifuged 5000g, 4°C, 5 min. They were transferred into 1.5 ml Eppendorf tubes containing Lysis Buffer A (Hepes KOH pH 7.8, 90 mM; KCl 0.8 M; EDTA 2 mM; glycerol 20% ; 0.3% Triton). The volume of Buffer A was chosen according to the lungs weight. The fragments were then disintegrated for 4x20 secs using a Qiagen Tissue Ruptor. Cytosolic and nuclear membranes were destroyed by 2 freezing (in liquid nitrogen)/thawing cycles. The whole cell extracts will then be cleared by centrifugation (16 000 g) for 5 min, 4°C. Extracts were aliquoted and stored at -80°C.

Four mice (M1 to M4) and four rats (R1 to R4) were sacrificed and the corresponding lung extracts were prepared.

3. Preliminary Repair activities' measurements

The aim of these preliminary experiments was:

- to get an idea of the inter-extract (inter-animal) variability
- to compare the repair activities between Mice and Rats
- to determine the best experimental conditions (extract concentration and reaction time) to be used for the Vanadium experiments (see Table 1).

Oligo Biochip

All extracts were tested at 4 different concentrations (Table 1). The digestion was conducted for 60 min at 37°C. All conditions were tested in duplicate.

We followed the protocol described in (Pons et al, 2010).

Plasmid Biochip

- Basic version

All extracts were tested at 5 different concentrations (**Table 1**). The repair reaction was conducted for 2H and 3H at 30°C in the presence of dCTP-Cy3 (Series 1).

All conditions were tested in duplicate.

We followed the protocol described in (Prunier et al, 2011).

Subsequently the experiments were repeated using only 2 extract concentrations and one repair time (Series 2).

- Klenow version

All extracts were tested at 5 different concentrations (**Table 1**). The Excision reaction was conducted for 30 min at 30°C.

After completion of the reaction, the slides were rinsed in H₂O, 2x3 min. The strand termini generated by the excision were then labelled by extension using DNA polymerase I, Klenow fragment (3'→ 5'exo-) and dCTP-Cy3. The reaction processed for 20 min at 37°C.

All conditions were tested in duplicate.

Table 1: Preliminary repair experiments – test of rodent extracts concentration and repair time

Rodent extracts	<i>Oligo Biochip</i>	<i>Plasmid Biochip</i> Basic version	<i>Plasmid Biochip</i> Klenow version
		Series 1	
Protein concentration	5, 10, 20 and 50 $\mu\text{g/mL}$	0.05, 0.1, 0.2, 0.5 and 2 mg/mL	0.005, 0.01, 0.02, 0.05, and 0.1 mg/mL
Repair reaction time	60 min 37°C	2h and 3h, 30°C	30 min, 30°C (extract) then 20 min, 37°C (Klenow)
		Series 2	
Protein concentration		0.2 and 0.5 mg/mL	
		2h, 30°C	

4. Preliminary Vanadium experiments

The exposure concentrations of vanadium pentoxide ultimately used were dictated by the concentrations of the vanadium element measured in the lung tissues of mice at the end of the reported 16-day inhalation experiment (Schuler et al., 2011). Based on the concentrations of vanadium measured in the lungs of mice in the 16-day inhalation study (8.02, 31.01 and 64.35 $\mu\text{g V/g lung}$) and the use of a conversion factor of 1.785 (i.e., ratio of the molecular and atomic masses: $\text{V}_2\text{O}_5/2\text{V} = 182/102$) these *in vitro* experiments should use an exposure range, as vanadium pentoxide, of 14 – 115 $\mu\text{g/ml}$ medium and, additionally include a vehicle or medium-only control and a positive control substance, cadmium chloride (CdCl_2).

The aim of these experiments was to set up the optimum experimental conditions for the determination of the effect of V_2O_5 on the DNA repair activities.

For this purpose, serial concentrations of the tested compound were incubated with the extracts on ice for different times (see **Table 2**) and then the mix was used for the repair experiments.

The experiments were conducted using the protein concentration and the repair reaction time selected from the preliminary repair experiments.

Experiments with Cadmium Chloride (Cd Cl_2) were run in parallel to serve as positive control for inhibitory effects (Candéias et al, 2010).

Both compounds were dissolved in H_2O .

V_2O_5 stock solution was prepared at 1 mg/mM (5.5 mM) and kept at 4°C.

CdCl_2 stock solution was prepared at 9.1 mg/mL (50 mM) and kept at 4°C.

Two series of ODN Biochip testing were performed: CdCl_2 concentration was increased for the second series.

Table 2: Preliminary vanadium experiments - experimental conditions tested

	<i>Oligo Biochip</i>	<i>Plasmid Biochip</i> Basic version	<i>Plasmid Biochip</i> Klenow version
Extract concentration	20 $\mu\text{g/mL}$	0.5 mg/mL	0.05 mg/mL
Incubation time (Extract+compound)	10, 20, 30 min 4°C	10, 20, 30 min 4°C	10, 20, 30 min 4°C
Repair Reaction	60 min, 37°C	2 h, 30°C	30 min, 30°C
<i>Compounds tested concentrations</i>			

V ₂ O ₅	µg/mL	0	9.375	18.75	37.5	75	150	300
	mM	0	0.05	0.10	0.21	0.41	0.82	1.65
CdCl ₂ (1)	µg/mL	0	5.7	22.8	91	First ODN Biochip and Plasmid Biochip test		
	mM	0	0.03	0.12	0.50			
CdCl ₂ (2)	µg/mL	0	100	200	300	Second ODN Biochip test		
	mM	0	0.55	1.09	1.64			

Results

1. Preparation of lung extracts

All preparations gave similar amount of proteins (mean= 11.44 ± 1.33 mg/mL). Aliquots of the extracts were prepared and stored at -80°C. They were used once and then discarded.

2. Preliminary Repair activities' measurements

Oligo Biochip

Observation: contrary to what is usually observed with human cell lines extracts, no activity against U paired with A was detected either with Mice or with Rat extracts. This might be specific for tissues. We have made the same observation on another strain of mouse (skin extracts) used for other applications.

More activities could be detected with the Rat extracts than with the Mouse extracts. In particular cleavage of EtheA, Hx, 8oxoG, A-8oxoG and Tg, were low or even absent with Mouse extracts whereas they were always detectable with Rat extracts.

The rate of cleavage of all lesions was related to the amount of protein.

A protein concentration of 20µg/mL was selected for the vanadium experiment as at 50 µg/mL non-specific activities were observed (i.e., degradation of the control DNA).

Plasmid Biochip

- Basic version

Excision/Synthesis activities in the lungs are rather low yielding low fluorescent signals on the Plasmid Biochip. We observed a rather homogenous signal on the Biochip, whatever the lesion investigated, especially with the Mice extracts, an observation that is not expected when enzymatic activities are specific.

In addition for certain lesions the repair signal was not proportional to the amount of protein. Results after 3h reaction were not improved over those obtained at 2h. So, a 2h reaction time was selected for the compound testing experiments.

We chose to perform the experiments with the compounds using a final protein concentration of 0.5 mg/mL. However, some non-specific activities (e.g., nucleases) might interfere with the results, especially with the Mice extracts.

- Klenow version

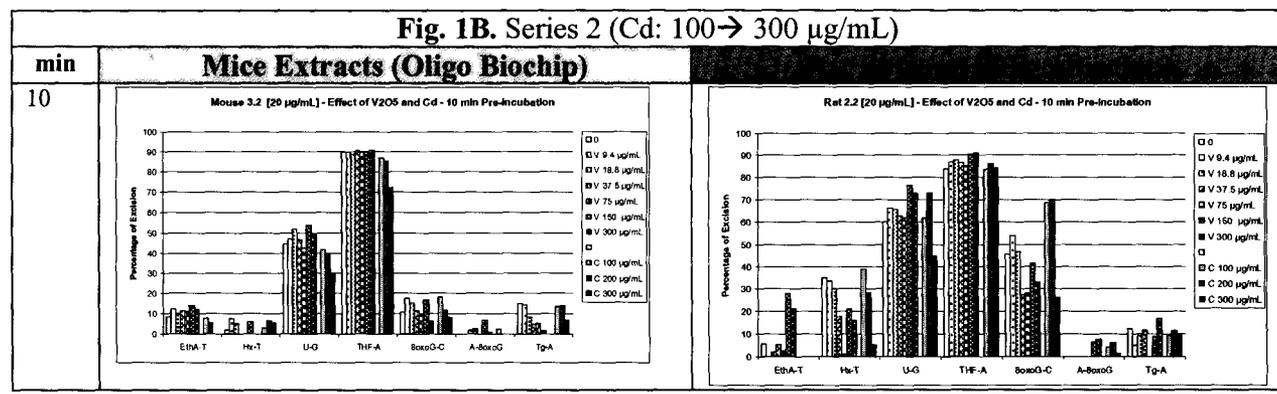
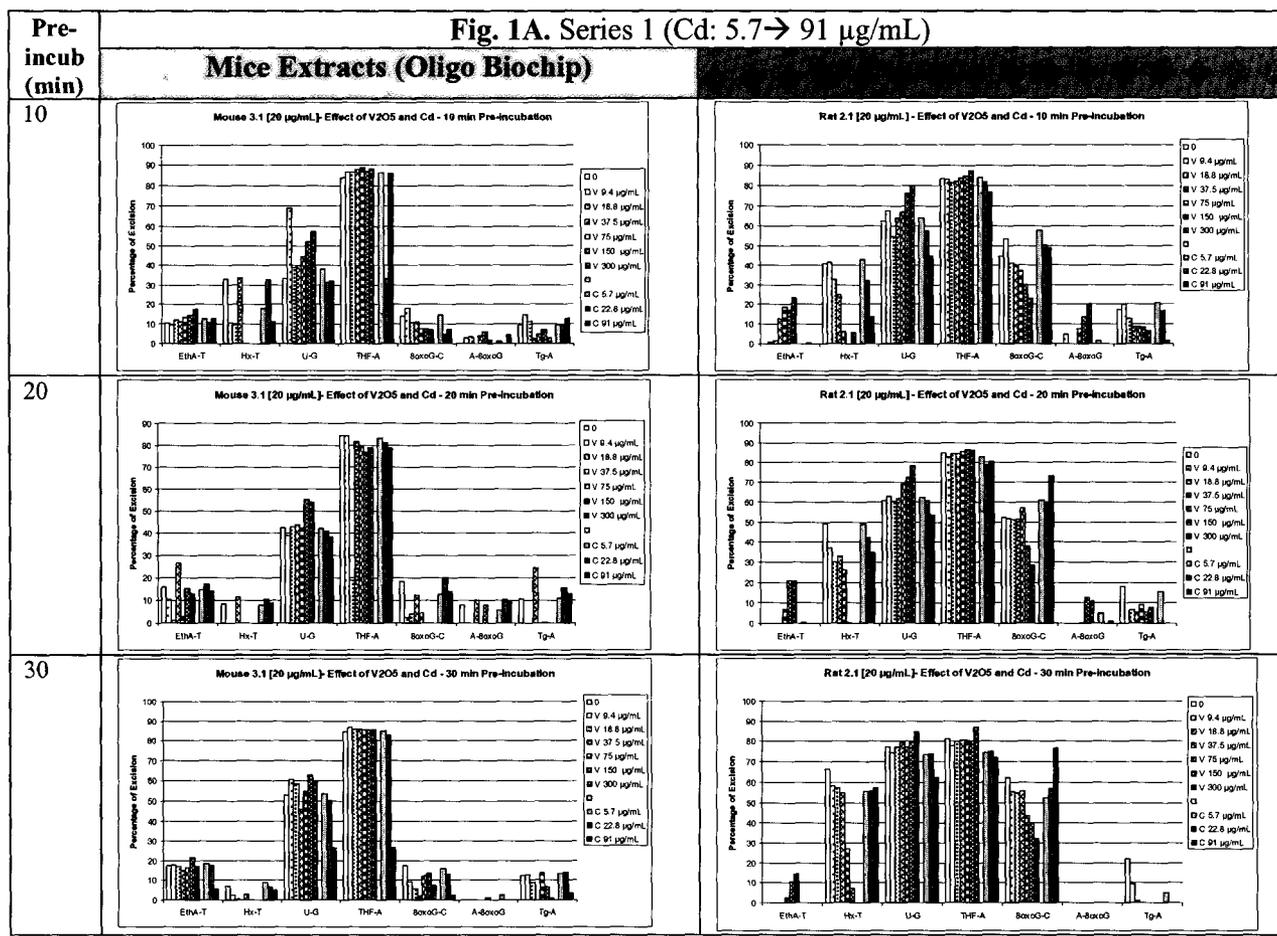
Excision activities were proportional to the amount of proteins except for M4 where an inverted relationship was observed. This extract was discarded.

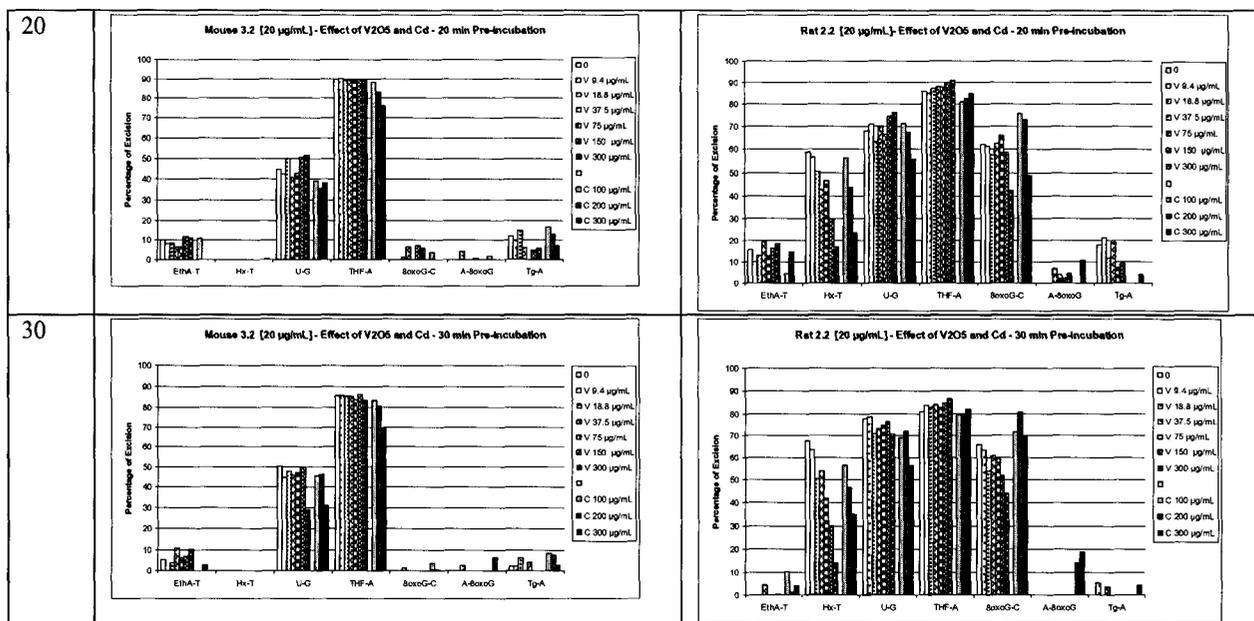
A concentration of 0.05 mg/mL was selected for the compounds testing.

3. Preliminary Vanadium Pentoxide experiments

These results are preliminary and all experiments must be repeated in order to confirm (or not) them.

Oligo Biochip (Figure 1A and 1B)





Comments for the Oligo Biochip results:

Series 1: the impact of V_2O_5 on Glycosylase/Lyase activities is detected from 10 minutes pre-incubation.

Paradoxical effects were noticed after 10 min pre-incubation. Whereas some excision activities decreased, other increased. Decreases were observed in the repair of 8oxoG, hypoxanthine (Hx), and to a lesser extent thymine glycol (Tg) whereas some increase was observed in the repair of EthA, Uracil. These effects were more obvious with the Rat extracts as repair activities were higher than with Mice extracts.

The negative impact of V_2O_5 on repair of 8oxoG and Hx was observed with Rat extracts whatever the pre-incubation time considered.

As results with Cd were inconsistent, a second series of tests was performed using the same V_2O_5 concentrations but increased Cd concentrations.

Series 2: although results of Series 2 were less marked than for Series 1, they seemed to confirm that V_2O_5 inhibited excision activities directed against 8oxoG and Hx for Rat extracts. The excision activities directed against 8oxoG were hardly detectable using Mice extracts.

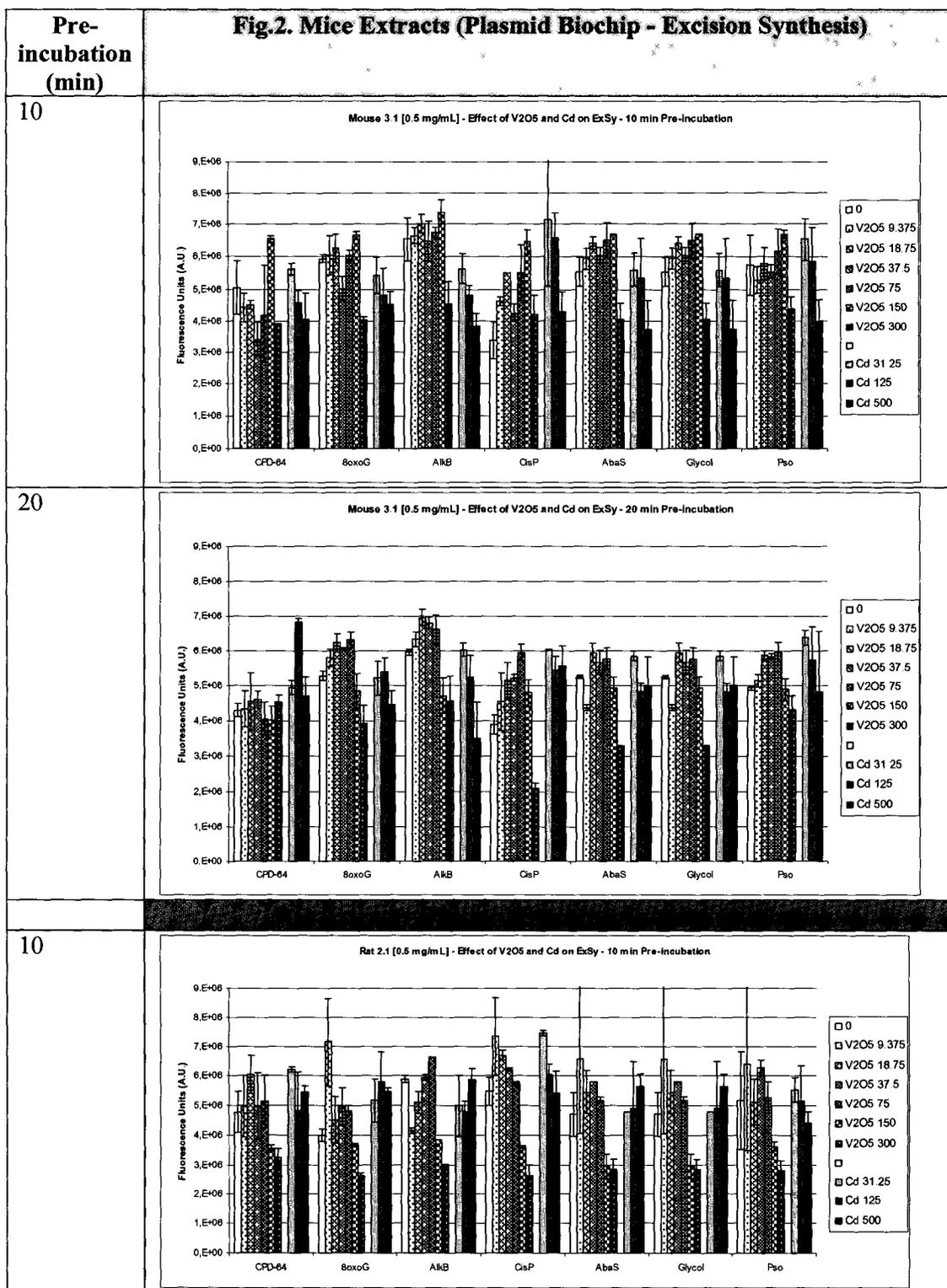
No effect of V_2O_5 on the cleavage of AP site was detected.

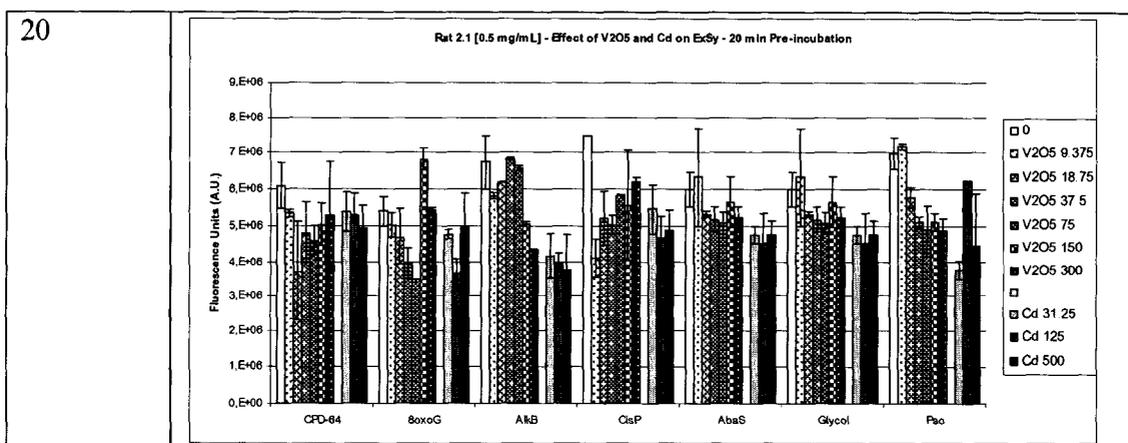
In order to confirm the effect of V_2O_5 on Excision of 8oxoG and Hx, this experiment will be conducted a third time with increased Cd concentrations, fresh V_2O_5 solution and changing a little bit the experimental procedure to avoid any possible bias.

Plasmid Biochip (Figure 2)

- Basic version

Although 3 incubation times between extracts and compounds were tested (10, 20 and 30 min), only results from the 10 and 20 min incubations are shown in Figure 2. This is because there were drastic reductions in activity at the 30 min incubation and consequently the signals were very low and not considered to be reliable.





Comments on the *Plasmid Biochip* (Basic version) results:

As initial fluorescent signals were low, the intra-experiment variability was high. Thus, the results must be taken with caution and the experiments will be repeated several times before we can give a definite conclusion.

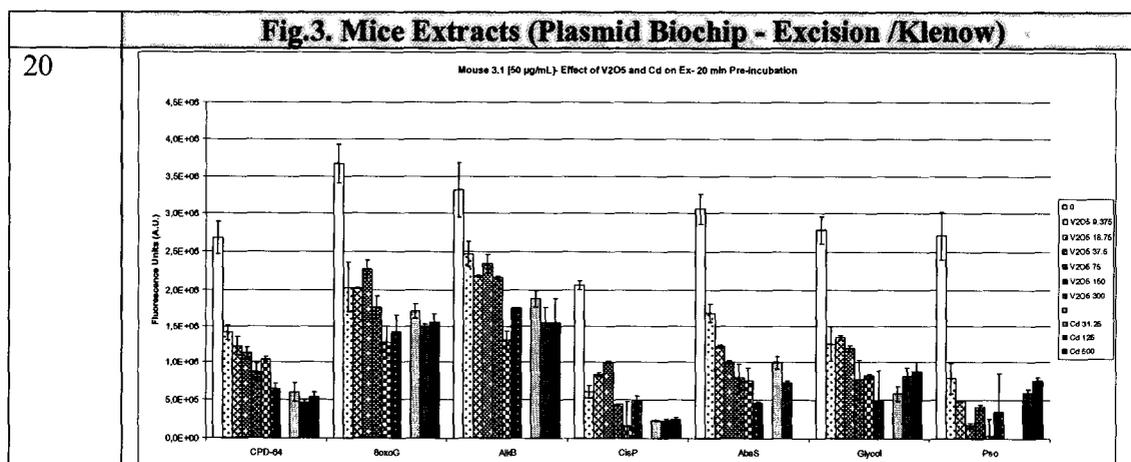
For Mouse extracts pre-incubated for 10 and 20 min, the highest concentration of V₂O₅ (300 µg/mL) led to significant decreases of the Excision/Synthesis repair activities whatever the repair pathway considered, with few exceptions.

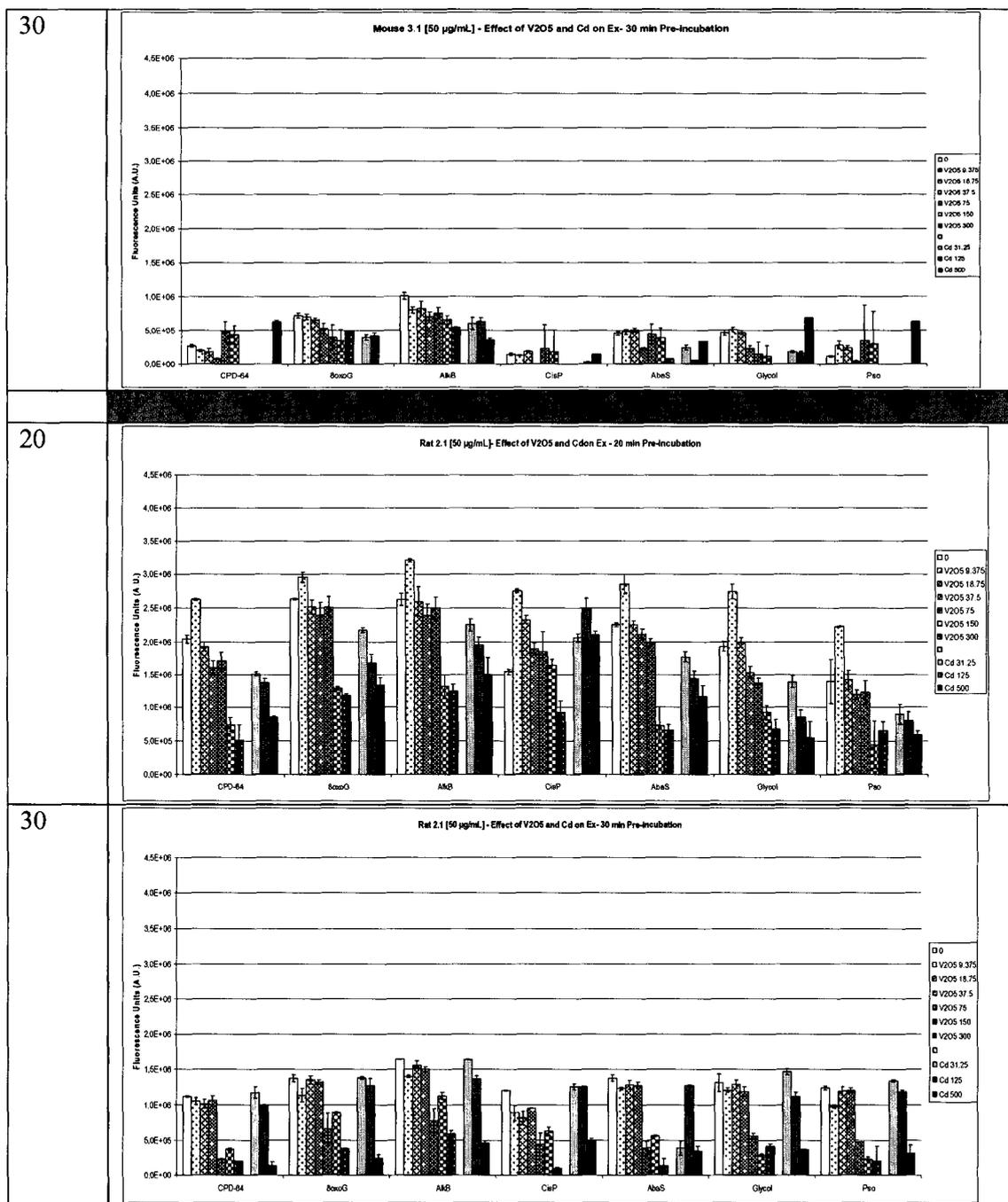
In the case of Rat extracts pre-incubated for 10 min, inhibition of Excision/Synthesis by V₂O₅ was observed at 150 and 300 µg/mL. Results after 20 min pre-incubation seemed not to produce any significant effects.

For the next experiments, the Cd concentration range used will be increased.

- Klenow version (Figure 3)

Two incubation times were tested up to now. The 10 min incubation time point is missing. The 30 min incubation point gave low signals. It seems the repair activities were not stable and decreased together with increased pre-incubation time.





Comments for the *Plasmid Biochip* (Klenow version) results:

There was a strong tendency toward a decrease of Excision activities against all lesions with increasing V₂O₅ concentrations. This seemed significant starting at 75-150 µg/mL (depending on pre-incubation duration).

For Rat extracts, the Cd results showed the same trend.

Preliminary conclusion

It was possible to quantify several DNA Excision and Excision/Synthesis repair activities belonging to Base Excision repair, Nucleotide Excision Repair and Intra-Strand Cross Link Repair using the 3 *in vitro* assays and Mouse and Rat lung extracts.

However the results that concerned the Excision/Synthesis capacity of Mouse extracts measured using the *Plasmid Biochip* must be taken with caution as the repair activities were low and some nucleases activities probably interfere with the signals.

From the preliminary experiments, it appeared that the V₂O₅ had the ability to interfere with several Excision activities in certain conditions (*Oligo Biochip*). This phenomenon was visible from 10 min pre-incubation.

In particular, Excision of 8oxoG (probably by OGG1) and Hx (theoretically by MPG although it seems unlikely here as MPG is also supposed to cleave EtheA for which different cleavage profile appeared) was inhibited by V₂O₅.

A better titration of the effect by V₂O₅ will be attempted in subsequent experiments.

It seems also that V₂O₅ had a negative impact on the Excision/Synthesis repair activities (*Plasmid Biochip*) although this was pre-incubation time-dependent and species-dependent and needs to be confirmed at least on Rat extracts.

Results obtained with the Klenow version of the *Plasmid Biochip* reinforced the suspicion that V₂O₅ induced a decrease of Excision activities toward most of the lesions.

For the next series of experiments the Cd concentration range will be increased as in the present results Cd effects are not always consistent.

In these later experiments, efforts will be concentrated on the 10 and 20 min pre-incubation times as repair signals were too low at the 30 min pre-incubation time point. It appears that the extracts are not stable and loose activities during this pre-incubation period.

As the V₂O₅ preliminary experiments were performed on extracts from one Mouse and one Rat, they will be repeated on extracts prepared from different animals to confirm the identified effects.

References.

Candéias S, Pons B, Viau M, Caillat S. & Sauvaigo S. (2010) Direct inhibition of excision/synthesis DNA repair activities by cadmium: analysis on dedicated biochips. *Mutat Res.*, **694**:53-59.

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Prunier C, Masson-Genteuil G, Ugolin N, Sarrazy F. & Sauvaigo S. (2011) Aging and photo-aging DNA repair phenotype of skin cells-Evidence toward an effect of chronic sun-exposure. *Mutat Res.* [Epub ahead of print]

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APPENDIX I: CERTIFICATE OF ANALYSIS

STRATCOR TECHNOLOGY CENTER

ANALYTICAL LABORATORY
HOT SPRINGS, ARKANSAS

Certificate Of Analysis



Vanadium Pentoxide - Batch H110435

(All assays in Percent)

Element	%	Element	%
V ₂ O ₅	100.33	Co	<.001
V ₂ O ₄	0.46	Cu	<.001
C	<.001	Ga	<.001
S	<.001	Ge	<.001
Al	0.001	Hf	<.001
As	<.001	In	<.001
B	<.001	Ir	<.001
Ca	0.001	Li	<.001
Cr	<.001	Nb	<.001
Fe	0.001	Os	0.001
K	<.001	Pb	<.001
Mg	<.001	Pd	<.001
Mn	<.001	Pt	<.001
Mo	<.001	Rh	<.001
Na	<.001	Sb	<.001
Ni	<.001	Sn	0.001
P	0.002	Sr	<.001
Si	0.003	Ta	0.002
Ag	<.001	Th	<.001
Au	<.001	Ti	<.001
Ba	<.001	Tl	<.001
Be	<.001	U	<.001
Bi	0.001	Y	<.001
Cd	<.001	Zn	<.001
N	0.002	Zr	<.001

Reported By: M. R. WoolleyDate: Apr 20, 2011