Mutagenicity of Cigarette Smoke Vapour Phase in the AMES Test

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For more than 30 years the Ames test is a widely used *in vitro* method for the determination of cigarette smoke condensate mutagenicity in bacteria. Recent Canadian regulation requires cigarette testing according to Health Canada method T-501 with five tester strains of *Salmonella typhimurium*, TA98, T100, TA102, TA1535 and TA1537. It is well known from literature that these strains respond differently to cigarette smoke condensates. The mutagenic effects of fresh whole smoke and vapour phase in this bacterial system are less well known.

In the present study the mutagenicity of fresh whole smoke and its vapour phase was tested. All five tester strains were directly treated with freshly generated whole smoke and vapour phase from a 13 mg tar American Blend cigarette. Smoke of up to 12 cigarettes was bubbled through 12 ml of buffered bacteria suspension. The smoke exposed suspensions were further processed using standard Ames plate incorporation techniques.

In comparison to the other tester strains, TA98 showed the highest response to condensate, whereas vapour phase mutagenicity could only be detected with strain TA100.

In order to further investigate vapour phase mutagenicity, TA100 only was used for tests with different experimental cigarettes, namely American Blend style with three different filters and tar yields as well as three single grade tobacco style, Burley, Virginia and Oriental cigarettes. Mutagenicity was expressed per cigarette and a per mg tar, respectively. Mutagenicity of fresh whole smoke was compared with effects of vapour phase and those measured with Cambridge filter condensates. It is obvious that only part of the fresh whole smoke is retained by the bacterial suspension. In order to get an estimate of the particulate matter trapped optical density was measured at 370 nm.

Fresh whole smoke mutagenicity in TA100 was higher than vapour phase mutagenicity. On the other hand, on a per mg particulate matter basis, Cambridge filter condensate mutagenicity was lower than whole smoke mutagenicity, possibly caused by some lack of vapour phase compounds in tar or different mutagenic mechanisms.

Significant differences were also found between cigarettes with different tar yields and tobacco types, Burley exhibiting the most pronounced effects, confirming the data from previous condensate studies.

Introduction

The biological effects of the cigarette vapour phase are gaining more and more importance (Aufderheide and Gressmann 2007). The Ames test, especially with *S. typhimurium* TA98 as a current in vitro test for mutagenicity, delivers reliable information about cigarette smoke condensate (CSC) mutagenicity. The method presented here enables determination of cigarette specific mutagenicity, in particular from the vapour phase. Simple equipment mainly consisting of three parts, a smoking machine, impinger and photometer is sufficient to do the test. The influence of vapour phase on the mutagenicity of the cigarette is an important point for the correct evaluation of products with regard to their in vitro mutagenicity.

Material and Methods

Experimental cigarettes

Three experimental cigarettes of the American Blend type and three single grade tobacco cigarettes according to table 1 and 2 were used in this study:

Code	Filter	Filter vent. [%]	Total Particulate Matter **TPM** [mg/cig]	water free Part. Matter **DPM** [mg/cig]	ISO-Nic [mg/cig]
AB-Tube	PE-tube, 21 mm	0	28.8	20.6	1.4
AB-0%V	acetate, 21 mm	0	13	11.6	0.7
AB-30%V	acetate, 21 mm	30	10.3	9.6	0.6

Table 1: Experimental cigarettes of the American Blend type

Code	Filter	Rod Weight [mg/cig]	Total Particulate Matter **TPM** [mg/cig]	water free Part. Matter **DPM** [mg/cig]	ISO-Nic [mg/cig]
Burley	acetate, 21 mm	650	13.4	12.3	0.8
Virginia	acetate, 21 mm	788	17.5	15.7	1.5
Orient	acetate, 21 mm	862	22.5	19.3	0.7

Table 2: Experimental cigarettes made from single grade tobacco (non-ventilated).

Prior to use the cigarettes were stored in a conditioning chamber at 22°C and 60% relative humidity.

Conditioning and smoking was performed under ISO conditions. For CSC testing only, Total Particulate Matter (TPM) collected onto a 92 mm Cambridge filter pad, extracted 30 minutes at room temperature with DMSO and initially frozen was tested. Nicotine and water in the TPM was analysed and the water free particulate matter (DPM) was calculated.

Preparation of bacteria suspension for Ames test

Before starting the test 10 ml cultures of *S.typhimurium* were incubated at 37°C overnight in antibiotic supplemented Nutrient Broth. After 15 hour additional 30 ml of fresh medium were added and for further 2 hrs incubated. The ready to use bacteria suspensions were prepared by centrifugation at 2300 x g for 10 minutes. The pellet was re-suspended in 40 ml of Ca, Mg free Dulbecco's PBS with 0.5% DMSO. The bacteria were prepared and treated at room temperature under protection from light.

Smoke treatment of bacteria

Aqueous vapour phase or whole smoke extracts were prepared using a 3 port adapter RM158 (Burghart Instruments, Wedel, Germany) connected with a single port smoking machine (ISO smoking regime). To minimise dead volume in the 92 mm diameter Cambridge filter holders a special holder with reduced inner volume of 14 ml was used.

Smoke of cigarettes yielding less than 13 mg DPM was bubbled in up to four consecutive runs with three cigarettes each through an impinger containing a tube with 12 ml bacteria suspension in PBS/DMSO. Smoke extracts prepared from 9 or more cigarettes with higher DPM yields were too toxic for a reasonable evaluation. In this case smoke of up to 8 cigarettes was bubbled in up to four consecutive runs of two cigarettes each. After each puff of each cigarette a flushing step with fresh air followed.

Ames Test

S. typhimurium strains TA98, TA100, TA102, TA1535 and TA1537 were originally furnished by Dr. Bruce Ames (University of California, Berkeley, CA). The Ames test was performed as recommended by Maron and Ames [1983]. For each Petri plate 100 µl of bacteria suspension were used. All mutagenicity assays were conducted in the presence of metabolic activation (S9).

Revertant colonies were counted using an automatic colony counter (ARTEK Counter; Model 880).

Negative and positive controls

Negative (DMSO) and positive controls (TA98: 2-Aminofluorene; TA100: 2-Aminoanthracene) were concurrently run with each test.

Determination of dry particulate matter retained by the bacteria suspension

The amount of particulate matter trapped in the medium was estimated in additional tests by measuring optical density (OD) at 370 nm. The smoke bubbled bacteria suspension was colorimetrically measured immediately after each run.

In order to determine DPM yields and DPM concentrations in the medium comparable with CSC DPM, gradients of known concentrations of CSC in DMSO and in TA100 in PBS/DMSO suspension, respectively, were prepared. The concentration [µg/ml] of particulate matter in whole smoke bubbled bacteria suspension was then calculated based on the calibration curves generated.

Calculation of mutagenicity

Initially, cigarette specific mutagenicity data were plotted as dose-response linear regressions with 95% confidence interval, mutagenicity [revertants per plate] vs. dose [cigarette equivalents per plate]. Data presented here refer to both, mutagenicity per cigarette, and per µg particulate matter (re-calculated).

Cigarette smoke condensate (CSC) specific mutagenicity was directly determined with plates containing known amounts of CSC per plate (standard procedure when testing CSC).

For a reasonable comparison of (standard) CSC specific mutagenicity and whole smoke or vapour phase mutagenicity it is necessary to know the percentage of particulate matter trapped by the smoke bubbled bacteria suspension (see above).

Based on the amount of particulates trapped by the bacteria suspension, new regression lines and new slopes [rev per µg trapped] were generated with GraphPad Prism 5.0. These data were used to calculate, based on standard CSC mutagenicity data [rev/µg], the cigarette specific mutagenicity (theoretically originating from trapped particulate matter), and compared with whole smoke and vapour phase mutagenicity data.

Results and discussion

Choice of medium for bacteria suspension

In order to identify the best matrix for mutagenicity testing, different suspensions of TA100 with AB-0%V cigarette were tested. Nutrient Broth (NB) as a typical medium for *Salmonella* strains guaranteed the best growth. However, the complexity of the medium could be a problem. For the tests, the bacteria should remain under consistent conditions for some hours. Metabolic waste products in NB might be influencing the interaction of smoke constituents with the bacteria. PBS buffer arrests the bacteria growth for a longer period of time. The addition of DMSO in PBS facilitates the absorption of smoke matter in the fluid medium.

The Ames tests with bubbled smoke in PBS/DMSO in comparison to fresh NB showed a more than 35% higher response. The concentration of DMSO in the PBS buffer was adjusted to 0.5%. Higher concentrations of DMSO decreased the response.

Screening of *S. typhimurium* strains for response to cigarette Whole Smoke (WS) and Vapour Phase (VP)

PBS/DMSO suspensions of five *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 were prepared. The response to bubbled WS and VP of AB-0%V test cigarettes was tested.

The response of strains was as follows:

Strain	TA98	TA100	TA102	TA1535	TA1537
Whole smoke	+	++	-	-	-
Vapour phase	-	++	-	-	-

Table 3: Response of *S. typhimurium* strains to whole smoke and vapour phase

Calculation of dry particulate matter concentration in the smoking system

As mentioned earlier, calibration curves of CSC in DMSO and TA100 in PBS/DMSO, respectively, were used to estimate the DPM yield and the amount of particulate matter trapped by the bacteria solution when bubbled with smoke. During each VP test with RM158, DPM yields of test cigarettes were determined by eluting the Cambridge filter placed before the impinger in DMSO, measuring the optical density (OD) and multiplying with the empirical 'DMSO factor' given in the table below.

The concentration of DPM trapped by the bacteria solution when testing whole smoke was determined by measuring its OD and employing the TA100/PBS factor. Percent amounts trapped by the suspension are given in the table below.

Cigarette	AB-Tube	AB-0%V	AB-30%V	Burley	Virginia	Orient
TA100/PBS-Factor [$O.D._{370nm}$ → µg/ml]	**1198**	**1209**	**1160**	**1396**	**1364**	**1265**
DMSO-Factor [$O.D._{370nm}$ → µg/ml]	**1836**	**1890**	**1872**	**2039**	**2079**	**2048**
DPM in TA100/PBS [%]	**3.64**	**2.21**	**1.07**	**2.57**	**2.46**	**3.35**

Table 4: DPM factors and percent of trapped DPM in bacteria suspension.

In addition, a 'smoke mass balance' of the system was done. When testing WS, 60% of DPM were found after the impinger, 20 to 25% on the glass material of impinger and test tube, and less than 15% had escaped the system.

Cigarette specific WS and VP mutagenicity

In order to determine mutagenicity of whole smoke and vapour phase three replicate tests were performed. The cigarette specific mutagenicity [revertants per cigarette] and 95% confidence intervals were calculated with GraphPad Prism 5.0. The calculation was based on cigarette equivalents per ml PBS bacteria suspension with 0.5 % DMSO.

The whole smoke mutagenicity with TA100 was higher as or similar to the vapour phase mutagenicity. Especially the high yield cigarettes with filters did not show significant differences between the WS and VP mutagenicity.

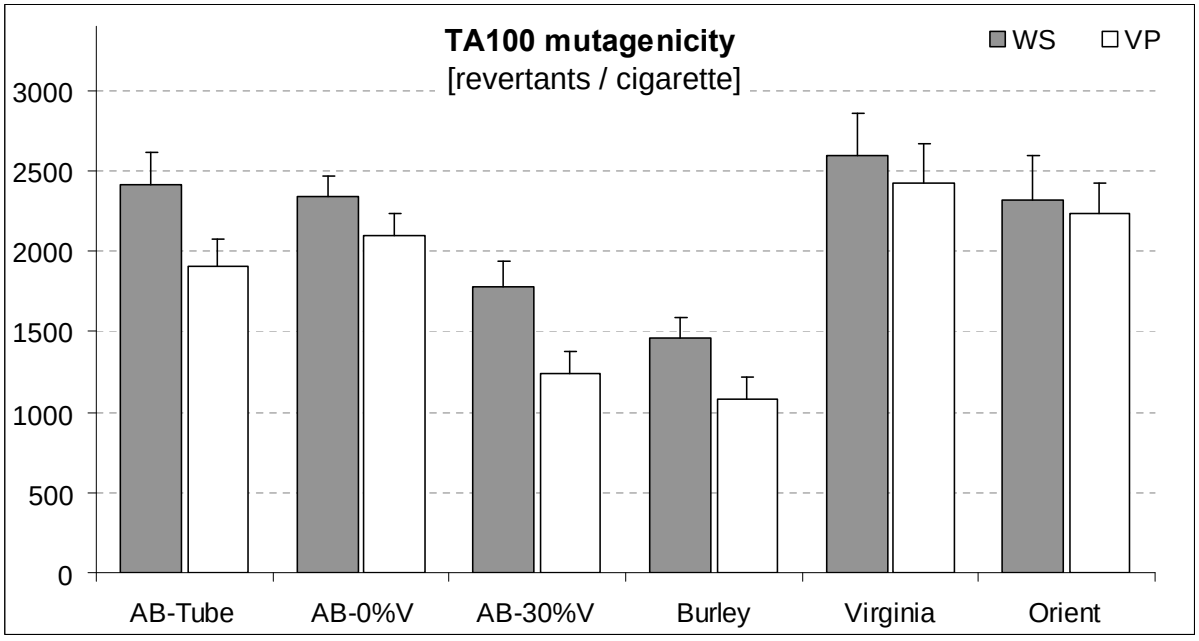

Fig. 1: Whole Smoke (▩ WS) and Vapour Phase (□ VP) cigarette specific mutagenicity with 95% confidence intervals.

Condensate (CSC) specific mutagenicity

In order to determine mutagenicity of CSC, three replicate tests were done with CSC collected on a 92 mm diameter Cambridge filter (RM 20) and dissolved in DMSO. The mutagenicity of DPM was calculated as described above. The DPM specific mutagenicity was similar for most cigarettes, but Burley cigarettes showed significantly higher mutagenicity. The theoretical cigarette specific mutagenicity was re-calculated via multiplication of DPM specific mutagenicity and DPM yield of the cigarettes.

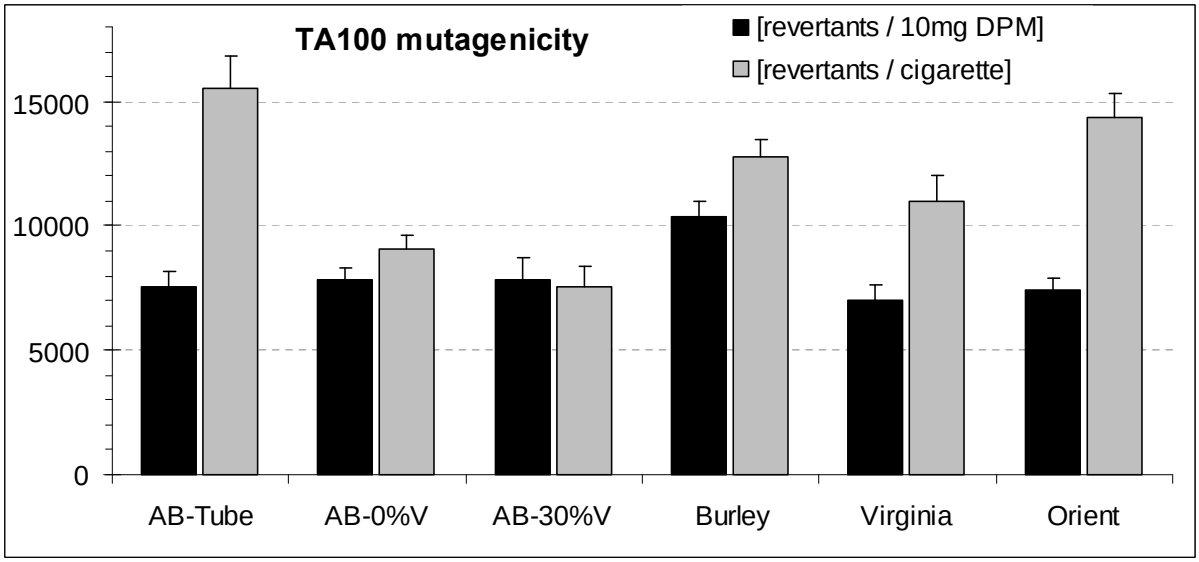

Fig. 2: Water free Particulate Matter (■ DPM) and cigarette specific (□ CS-DPM) mutagenicity with 95% confidence intervals.

Cigarette specific mutagenicity as calculated from routine CSC testing was much higher in comparison to the directly measured response to fresh whole smoke. The differences are caused by the limited solubility of particulate matter in the bacteria suspension. It is assumed that other smoke components as well are only partially solubilised in the fluid medium.

Comparison of mutagenicity data

It was obvious that only part of whole cigarette smoke mutagenicity originated from particulates. The DPM concentrations in bacteria suspensions were determined. The percent amount of trapped condensate was directly dependent on DPM yield. In the bacteria suspension exposed to smoke of AB-30%V cigarette, only 1.1 % of the whole DPM was found. With 3.6% trapped DPM, the AB-Tube cigarettes showed the highest amount.

The cigarette specific mutagenicity, derived from the amount of trapped particulate matter in the suspension, showed some differences in comparison to the directly calculated DPM cigarette specific mutagenicity based on standard CSC testing (data not shown).

The effects of WS, VP and trapped particulates in the bacteria suspension are shown in the diagram. For ease of comparison, the relative changes of the cigarette specific mutagenicity are shown (AB-Tube cigarette=1). Additionally, relative changes DPM and VP carbonyls were plotted in the same scale.

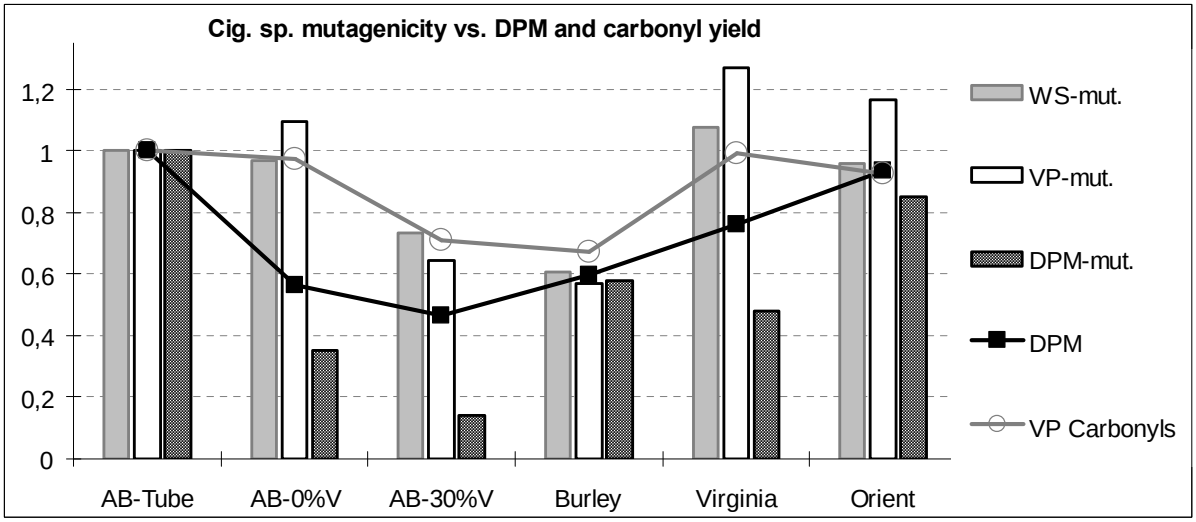

Fig. 3: Cigarette specific mutagenicity versus DPM yield and total vapour phase: Whole Smoke mutagenicity (▒ WS-mut.), Vapour Phase mutagenicity (□ VP-mut.), DPM mutagenicity calculated trough O.D.$_{370nm}$ (■ DPM-mut.), DPM yield (▪ DPM) and carbonyls in VP (⊖ VP Carbonyls).

Comparison of AB-0%V cigarette specific mutagenicity in TA100 and TA98

For the AB-0%V cigarette, whole smoke, vapour phase and standard CSC mutagenicity were determined with both TA100 and TA98. The revertants per cigarette were calculated and compared. In the smoke bubbled bacteria suspension 2.21 % of the cigarette DPM was trapped. The corresponding contribution of trapped DPM on mutagenicity was calculated.

The TA100 mutagenicity of vapour phase was 10% lower than whole smoke mutagenicity, and the particulates theoretically contribute 9% of whole smoke mutagenicity. With TA98 no vapour phase mutagenicity was detected. The mutagenicity contributed by particulates fully corresponded with whole smoke mutagenicity.

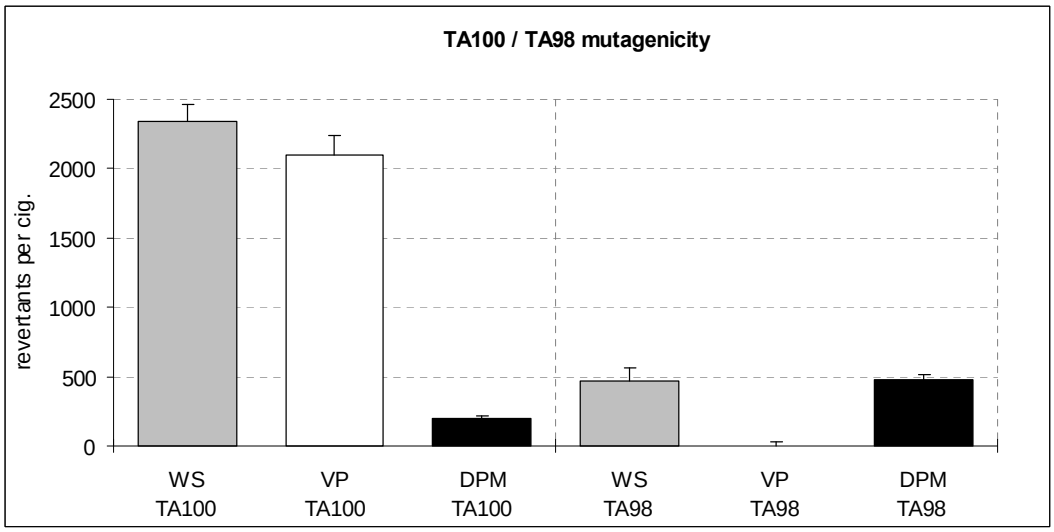

Fig. 4: AB-0%V cigarette specific mutagenicity (TA100 and TA98): Whole Smoke mutagenicity (▒ WS), Vapour Phase mutagenicity (□ VP), DPM mutagenicity calculated trough OD$_{370nm}$ (■ DPM).

Conclusions

Five strains of *Salmonella typhimurium* were tested for their response to fresh cigarette smoke in medium. Only strain TA100 showed condensate as well as whole smoke and vapour phase effects. Dependent on filter and tobacco type, 70 up to 96% of measured mutagenicity came from the vapour phase.

As can be seen in Fig. 3 the WS mutagenicity theoretically caused by trapped DPM correlated with the DPM yield of the cigarettes. The alteration of whole smoke and vapour phase mutagenicity correlated with the relative change of carbonyls in the vapour phase. Especially the alteration of vapour phase mutagenicity may be effected by the amount of acrolein in the smoke.

Fig. 4 very apparently shows differences between measured mutagenicity with TA98 and TA100. The whole smoke treated TA98 bacteria suspension solely shows WS mutagenicity derived from particulate matter, whereas *S. typhimurium* TA100 whole smoke mutagenicity consists of 10% from particulate matter and 90% from the vapour phase. However, when testing whole smoke, the proportion of vapour phase mutagenicity may change depending on the type of the cigarettes used. Therefore, the test with TA100 presented here should preferably be used for testing the vapour phase mutagenicity of cigarettes.

No significant cigarette specific differences were found between non-ventilated filter and no filter cigarette (tube instead of filter). The 30% ventilated cigarette showed a corresponding 30% lower mutagenicity. The Burley cigarette showed ca. 40% lower mutagenicity in comparison to Virginia and Orient.

CSC specific mutagenicity of the Burley as measured by standard testing was found to be highest, confirming previous research.

References

Aufderheide, M. and Gressmann, H. (2007): A modified Ames assay reveals the mutagenicity of native cigarette mainstream smoke and its gas vapour phase. *Exp Toxicol Pathol*. 58: 383-392.

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