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Mini mutagenicity test: a miniaturized version of the Ames test used in a prescreening assay for point mutagenesis assessment

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Abstract

The bacterial reverse mutagenicity test on *Salmonella typhimurium*, known as the Ames test, is widely used by regulatory agencies, academic institutions and chemical companies to assess the mutagenic potential of raw compounds. Several attempts have been made to miniaturise the Ames test in order to fit the industrial constraint of screening more products at the low quantities available. The major limitation of these miniaturised versions of the Ames test lies in the impossibility to work with all the six strains used in the regular Ames test, especially with those showing a low spontaneous revertant frequency. We describe here a mini version of the regulatory Ames test protocol that allows a significant reduction of the quantity of test substance needed (300 mg) but remains applicable to all *Salmonella* strains used in the regulatory protocol. In a preliminary study, 10 in-house chemical compounds have been evaluated in the Mini Mutagenicity Test (MMT) together with some positive control substances. A first set of historical data obtained in 1999 as well as the predictivity and the sensitivity of the MMT are presented and compared to those of the regular Ames test.

Keywords: Mutagenicity testing; Ames test; Miniaturisation

1. Introduction

Although some chemical carcinogens are non-genotoxic in conventional assays, DNA damage is considered as the initiating event by which a molecule causes hereditary effects (point mutation or chromosomal damage) and cancer (Venitt and Parry, 1984). Thus, the evaluation of the genotoxic potential of newly synthesized molecules constitutes one of the very important preliminary steps in the course of the safety assessment and regulatory control of chemicals. This evaluation is done mainly through in vitro assays.

The in vitro assessment of the genotoxic potential of a chemical is a multiple-step process requiring a "funnel-shaped" screening strategy based on a battery of tests (Agapakis-Caussé, 2001) from bacteria to mammals, in order to assess point mutations as well as chromosomal damage. Upstream of such a strategy are the tests based on bacterial systems which allow a rapid (thanks to the growth rate of bacteria) and relatively straightforward way (thanks to the biochemical selection of the mutants) to assess mutagenic potential. The bacterial reverse mutagenicity test on *Salmonella typhimurium* strains developed in 1975 by Bruce Ames (Ames et al., 1975) (also called the Ames test) belongs to the tests required by regulatory authorities and is usually used as an initial screen. This test is designed for the detection of the mutagenic potential of test substances through the induction of reverse mutations in the *His* gene of modified *S. typhimurium* strains.

For several years, a trend has been shown in the chemical industry in the intensification of the synthesis of new molecules to increase the chance of developing a lead compound. This increase in the number of molecules synthesised is usually accompanied by a reduction in the quantity of compound available. This imposes a new constraint on the toxicologist: the development of a

Abbreviations: DMSO, dimethyl sulfoxide; MMS, methyl methanesulfonate; MMT, Mini Mutagenicity Test; 4-NQO, 4 nitroquinoline *N*-oxide.

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miniaturised version of the tests in order to (i) evaluate more molecules in one single experiment and (ii) use a smaller quantity of the test compound to perform the test.

Different attempts to miniaturise the Ames test have been developed either on solid media (a direct plating test on a 24-well plates for the miniscreen test (Brooks, 1995) or in liquid medium in a 96-well plate format (Ames II^{TM} Xenometrix; Gee et al., 1998). The main disadvantage of using these versions is that either not all *Salmonella* strains can be used — especially, those which exhibit a low spontaneous revertant rate, such as the strains TA 1535 and TA1537 — for the miniscreen test (Burke et al., 1996) or non-conventional *Salmonella* strains are employed as in the Xenometrix® systems.

We describe here a miniaturised version of the Ames test: the Mini Mutagenicity Test (MMT), which is based on the standard regulatory Ames test protocol, (further referred to as the Ames test), but which allows a significant reduction in the quantity of test compound needed and enables the use of all classical *S. typhimurium* strains of the regular Ames test. This MMT has been performed in our laboratory for more than a year and permits the evaluation of the mutagenic potential of three test substances on six strains of *S. typhimurium* in the same experiment, or of more than three test substances on a reduced panel of strains, in a pre-screening assay for mutagenicity.

Compared to the standard Ames test, the MMT requires five times fewer reagents and is performed in six-well plates. These are more convenient to manipulate and all recommended treatments can be done: direct plate incorporation with and without metabolic activation system or pre-incubation. The scoring of the plates is easy and can be performed with an image analysis system or manually.

In a preliminary study presented here, nine in-house test substances plus two well known mutagenic chemicals were tested in parallel in the Ames test and in the MMT. Both tests, the Ames test and MMT, were performed in parallel on one or more strains in order to increase historical data, evaluate MMT variability and evaluate MMT predictivity

2. Materials and methods

2.1. Chemicals

Media and buffer for the Ames test and the MMT were prepared as described previously (Maron and Ames, 1983) with chemicals from Difco or Merck.

The positive controls used were 1,2 diamino-4-nitrobenzene [Sigma D6760, solvent–dimethyl sulfoxide (DMSO)] for strains TA1537/TA1538/TA98/TA100 without metabolic activation by S9 fraction, mitomycin C (Sigma M0503, solvent–water) for strain TA102 without metabolic activation, sodium azide (Sigma S8032, solvent–water) for strain TA1535 without metabolic activation, and 2-amino-anthracene (Sigma A1381, solvent–DMSO) for strains TA1535/ TA1537/ TA1538/TA98/TA100 in the presence of metabolic activation. As all the experiments in the presence of metabolic activation were conducted in parallel on the same day with all strains, we used mitomycin C as positive control for TA102 with S9 mix to avoid the use of benzo[*a*]pyrene, even though mitomycin C is a direct mutagen. We consider that the activity of the S9 mix is sufficiently controlled in the parallel experiments on the other strains with 2-amino-anthracene.

The test substances were chemicals (99.9% pure as certified by in-house analytical department) synthesized in the chemistry Department of the Advanced Research Laboratories at L'OREAL, Aulnay-sous-bois, France, and coded substance numbers one to nine and two well-known mutagenic chemicals: 4 nitroquinoline *N*-oxide (4-NQO, Sigma N8141) and methyl methanesulfonate (MMS, Sigma M4016).

2.2. S. typhimurium strains

Strains TA98, TA100, TA1535, TA1537 and TA102, recommended by the OECD guidelines, were used in this study. In addition, strain TA1538 was used in our laboratory as a complement in particular studies. The strains were purchased from the laboratory of Dr. B. Ames (University of California, Berkeley, CA, USA), and their genetic backgrounds (Ames et al., 1973; Levin et al., 1982) were controlled as described (Maron and Ames, 1983) every 6 months.

2.3. Ames test: treatment and plating

The Ames test was performed according to the OECD guidelines (OECD 471, 1997). Three different treatments were done: without metabolic activation (−S9), with metabolic activation (+S9) and with extended metabolic activation (pre-incubation protocol PI) of 1 h. The direct plating test protocol (Maron and Ames, 1983) was used: the bacterial strains were exposed to a range of concentrations of the test compounds in the absence or the presence of S9-mix in a soft agar overlay.

Briefly, for each petri plate, 100 μl of a 13-h culture of the strains were mixed in 2 ml of top agar with the 100 μl of test compound formulation and, for the activated tests, with 500 μl of S-9 mix in the following order: test compound, top agar, bacteria, S-9 mix (activated assay only). The tubes were swirled and poured on 90-mm petri plates. The culture plates were incubated at 37°C for 48 or 72 h (depending on the strains).

2.4. MMT: treatment and plating

The MMT was performed strictly with the same protocol as the Ames test (preparation of cells, treatment) except that all reagent volumes were divided by five. Briefly, 80 μl of the bacterial culture were mixed in 1.6 ml of top agar with 80 μl of test compound dilution and, for the activated tests, with 400 μl of S9 mix. After mixing 540 μl of the resulting mix were poured into six-well plates (35-mm dishes) containing 5 ml of minimal media. As soon as the soft agar solidified, the six-well plates were incubated at 37°C for 48 h.

One six-well plate was used for each dose level tested (Plate 1). The first three wells were used for the assay without metabolic activation, three other wells for the assay with metabolic activation (S9-mix).

2.5. Concentration range

Seven concentrations were tested in the first test:

1. For non-toxic and very soluble compounds, the highest concentration used was 1000 μg/well for the MMT and 5000 μg/plate for the Ames test.
2. For non-toxic and slightly soluble compounds, the highest concentration used was the lowest dose provoking a slight precipitate when added to the top agar (the presence of precipitate should not hinder scoring of revertants).
3. For toxic compounds, irrespective of solubility, the top concentration used was that causing moderate thinning of the bacterial background lawn (checked under a magnifying glass) and/or reduction in the number of revertants close to 75% as compared to the solvent controls.

The concentrations tested were: 5000, 2500, 1000, 500, 250, 100 and 20 μg per plate in the standard Ames test; 1000, 500, 200, 100, 50, 20 and 4 μg per well in the MMT.

For the second assay, five concentrations were tested without and with metabolic activation.

According to the results obtained in the first test in the presence of metabolic activation, the second test (repetition of the test) was either performed using a standard plate-incorporation technique or extended using the pre-incubation (37°C for 60 min) modification of the assay.

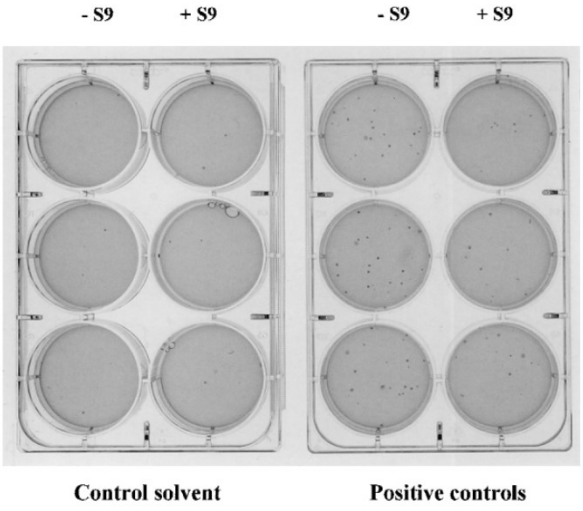

Plate 1. Picture of a typical MMT experiment on a six-well plate, on strain TA1535.

2.6. Preparation of the S9-mix

The S9 (rat, Arochlor 1254-induced) were purchased from Moltox™ (Molecular Toxicology Inc., Boone, NC, USA). The S9-mix (10% S9, v/v) was prepared shortly before use in a glucose-6-phosphate and NADP system according to Maron and Ames (1983).

2.7. Scoring and positivity criteria

The revertants obtained at each concentration and treatment were scored manually in the Ames or the MMT test. The acute observation, under a magnifying glass, of the bacterial lawn in each culture dish was used to check for possible toxicity of the treatment.

The expression and the input of the results were made as for the classical Ames test. The ratio (referred to below as "Mutagenicity ratio R" in the text, and "R" in the tables and figures) between the number of revertants at a given concentration and the revertants in the corresponding solvent control were calculated. A compound was considered to be mutagenic if this mutagenicity ratio was $R \geqslant 3$ for strains TA1535, TA1537 and TA1538, and $R \geqslant 2$ for strains TA98, TA100 and TA102. In both cases, a concentration-dependent response had to be observed. The same criteria for positivity were applied to the results obtained in the MMT test.

3. Results

3.1. Historical data: spontaneous revertants, solvent controls

The MMT has been developed in our laboratory since 1998 and extensively used from 1999. Thus, historical data from 30 independent experiments performed during the year 1999 were gathered and compared to those obtained in the regular Ames test.

Table 1 compares the historical data obtained in the MMT to those obtained in the Ames test with all six *Salmonella* strains, and in particular for strains TA1535 and TA1537, which exhibit the lowest spontaneous rate in the Ames test. The results demonstrate that the

Table 1
Historical values obtained for each Salmonella strain in the Ames test and in the MMT[ab]

	Solvent–water				Solvent–DMSO				Positive control			
	AMES		MMT		AMES		MMT		AMES		MMT	
	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.
TA 1535												
−S9	17	4	3	1	16	6	3	1	166	69	32	18
+S9	16	5	3	1	14	2	2	1	188	83	26	12
PI	15	5	2	1	13	3	2	1	140	57	25	12
TA 1537												
−S9	15	5	2	1	14	5	3	2	183	84	30	18
+S9	18	5	4	2	14	4	2	1	214	139	30	14
PI	15	4	4	2	15	4	3	2	180	91	29	19
TA 1538												
−S9	16	6	3	1	15	7	4	1	617	239	100	40
+S9	24	7	6	4	19	7	5	2	1400	852	203	82
PI	21	4	5	2	23	7	8	4	846	519	236	112
TA 98												
−S9	39	9	10	3	37	9	9	4	429	172	87	58
+S9	42	11	12	5	39	12	11	33	743	429	74	32
PI	43	11	11	3	41	12	10	4	1483	621	159	101
TA 100												
−S9	178	49	32	9	162	42	30	2	807	320	156	63
+S9	187	67	30	8	153	23	26	6	1103	681	118	42
PI	170	34	33	6	156	36	28	6	1654	672	158	66
TA 102												
−S9	425	108	86	25	420	114	71	23	2334	545	488	176
+S9	483	98	90	32	462	127	68	30	2103	577	501	174
PI	496	140	95	25	538	111	65	23	1851	603	391	114

[a] −S9, without metabolic activation; +S9, in the presence of metabolic activation; PI, in the presence of metabolic activation and pre-incubation protocol; M, mean; S.D., standard deviation.
[b] Values are the mean of 30 experiments.

miniaturisation of the test in the MMT protocol still enables the scoring of spontaneous revertants even for these two strains.

The miniaturisation resulted in a 3.5- to 5-fold reduction in the number of spontaneous revertants as compared to the regular Ames test, for strains TA1535 and TA1537 as well as for all the other strains used in this study (Table 1). It is noteworthy that strain TA98 showed the smallest reduction of spontaneous revertant rates as compared to the Ames test (mean of 3-fold reduction).

3.2. Historical data: revertants after treatment with positive controls

In the positive controls, treatment resulted in a significant increase in the number of His^+ revertants in the MMT. As previously observed for the spontaneous revertants, the miniaturisation results in a fivefold reduction of the number of revertants in the positive control as compared to the Ames test for strains TA1535 and TA1537 as well as for the other strains (Table 1).

Table 2 presents the comparison of the mutagenicity ratio *R* obtained for each strain after treatment with the positive controls in the MMT vs the regular Ames test. The mutagenicity ratios *R* calculated appear to be unaffected by the miniaturisation of the test: the ratios obtained in the MMT and in the regular test are comparable. Only for strain TA98, a slightly smaller ratio is obtained in the MMT as compared to the Ames test (Table 2). This is probably connected with the previous observation that TA98 is also the strain for which the smallest reduction in the number of spontaneous revertant is observed in the MMT.

3.3. Comparison of results obtained with the nine in-house test substances in the MMT and Ames test

Table 3 shows the results obtained for all *Salmonella* strains and the 11 substances tested (P1-P9, 4-NQO and MMS) in the MMT and in the Ames test. The same criteria for positivity were applied in the Ames and MMT, namely, a dose-dependent and biological significant increase in the number of revertants has to be observed.

Five substances (P1, P2, P3, P4 and P8) did not induce a biological significant increase in the number of revertants at any of the test concentrations and in all *Salmonella* strains used (Table 3); either in the regular Ames test or in the MMT.

In the regular Ames test, three substances (P5, P6 and P9) were found to induce a dose-dependent and

Table 2
Mutagenicity ratio obtained after treatment by positive control: comparison of MMT with the Ames test[a]

	TA1535			TA1537			TA1538			TA98			TA100			TA102		
	−S9	+S9	PI	−S9	+S9	PI	−S9	+S9	PI	−S9	+S9	PI	−S9	+S9	PI	−S9	+S9	PI
Ames test																		
R/water	10	12	9	12	12	12	39	58	40	11	18	34	5	6	10	5	4	4
R/DMSO	10	14	11	13	15	12	41	73	37	12	19	36	5	7	11	6	5	3
MMT																		
R/water	11	9	13	10	8	7	33	34	47	9	6	14	5	4	5	6	6	4
R/DMSO	10	11	13	10	13	10	28	40	30	10	7	15	5	4	6	7	7	6

[a] *R*/Water, mutagenicity ratio as compared to the solvent control water; *R*/DMSO, mutagenicity ratio as compared to the solvent control DMSO.

Table 3
Results obtained for the 11 chemical substances tested in the MMT vs the Ames test[a]

	TA 1535			TA 1537			TA 1538			TA 98			TA 100			TA 102		
	−S9	+S9	PI	−S9	+S9	PI	−S9	+S9	PI	−S9	+S9	PI	−S9	+S9	PI	−S9	+S9	PI
P1																		
AMES	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MMT	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P2																		
AMES	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MMT	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P3																		
AMES	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MMT	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P4																		
AMES	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MMT	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P5																		
AMES	– (Cyt. 2500)	– (Cyt. 2500)		– (Cyt. 2500)	– (Cyt. 2500)		– (Cyt. 2500)	3.4 (1000)		– (Cyt. 2500)	2.5 (1000)		– (Cyt. 2500)	– (Cyt. 2500)		– (Cyt. 2500)	–	
MMT							– (Cyt. 500)	3.4 (200)		– (Cyt. 500)	2.1 (100)					– (Cyt. 500)	–	
P6																		
AMES							– (Cyt. 2500)	2.8 (1000)		– (Cyt. 2500)	2 (1000)					– (Cyt. 2500)	–	
MMT							– (Cyt. 500)	2.8 (100)		– (Cyt. 200)	2.4 (50)					– (Cyt. 500)	–	
P7																		
AMES	–	–	–	–	–	–	–	–	–	–	–	–	1.5 (5000)	1.6 (2500)	2.1 (5000)	–	–	
MMT	–	–	[3] (Cyt. 500)	–	–	– (Cyt. 1000)	–	–	–	–	–	–	–	–	3.1 (500)	–	–	
P8																		
AMES	– (Cyt. 2500)	– (Cyt. 2500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	– (Cyt. 1500)	(Cyt. 1500)
MMT	– (Cyt. 250)	(Cyt. 1000)		– (Cyt. 250)	(Cyt. 1000)		– (Cyt. 250)	(Cyt. 1000)										
P9																		
AMES							–		3.3 (2500)	–		1.5 (5000)						

(*Table continued on next page*).

Table 3 (*continued*)

	TA 1535			TA 1537			TA 1538			TA 98			TA 100			TA 102		
	−S9	+S9	PI	−S9	+S9	PI	−S9	+S9	PI	−S9	+S9	PI	−S9	+S9	PI	−S9	+S9	PI
MMT							–	[2.8] (500)	3.7 (500)	–	[2.2] (500)	2.2 (250)						
MMS																		
AMES	–	–	–	–	–	–	–	–	–	–	–	–	3.3 (100)	2.6 (100)	–	2.4 (100)	2.3 (100)	
MMT	–	–	–	–	–	–	–	–	–	–	–	–	2.1 (20)	3.2 (2)	–	2.7 (20)	2.3 (20)	
4-NQO																		
AMES	3.5 (1)	7.3 (50)		– (Cyt. 5)	– (Cyt. 50)		2.6 (0.1)	8.7 (10)		8.4 (0.5)	9.1 (20)		11.7 (0.5)	17.8 (5)		8.7 (1)	2.7 (50)	
MMT	3 (1)	6 (10)		– (Cyt. 2)	– (Cyt. 10)		14 (0.02)	36 (0.7)		14 (0.02)	36 (2)		12.5 (0.02)	38 (2)		17 (1)	2.5 (6)	

[a] Compound non-mutagenic in the test. (Cyt. 2500): cytotoxicity observerd at a concentration of 2500 mg/plate. **2** (1000): compound presents a mutagenic potential, with a maximum ratio of mutagenicity (as compare to the solvent control) of $R = 2$. (1000) First statistically significative concentration.

biologically significant increase in the number of revertants of TA98 and TA1538 (Table 3, Figs. 1, 2 and 4, respectively), one compound P7 was found to be mutagenic on strain TA100 in the presence of metabolic activation with the pre-incubation protocol (Fig. 3). These four compound were classified as mutagenic in the Ames test. In the MMT, the same three substances were also mutagenic on the same strains. The well-known mutagen MMS was mutagenic in the standard Ames test on strains TA100 and TA102 (Fig. 5), as previously described in the literature (Levin et al., 1982). It also shows mutagenic activity on the same strains in the MMT. 4-NQO is mutagenic in the standard Ames test on strains TA1538, TA98, TA100 and TA102, in the absence or in the presence of metabolic activation (Fig. 6). The same results are obtained in the MMT, thus demonstrating good concordance with the results already described (Ames et al., 1973).

It is worth noting that in almost all cases the mutagenic activity is observed in the MMT at lower concentrations as compared to the Ames results (Table 3, Figs. 1–4). For instance, product P5 is mutagenic at a concentration of 1000 μg per plate in the Ames test (mutagenicity ratio $R = 2.1$) and is found to be mutagenic at a concentration of 100 μg per plate in the MMT ($R = 2.1$). For all the compounds that were mutagenic in the Ames test, the threshold for mutagenicity of the compound in a given strain was usually by a factor of about five lower in the MMT. This is confirmed by the study of the two well-known mutagens MMS and 4-NQO (Table 3, Figs. 5 and 6).

In summary, five compounds were found clearly non-mutagenic in the Ames test. These compounds were also non-mutagenic in the MMT. Six compounds were found to be mutagenic in the Ames test, they were all found to be mutagenic on the same strains in the MMT (Table 3). No false negatives were obtained in the MMT in this limited panel of test compounds.

4. Discussion

The Ames bacterial reverse mutation test enables the detection of the potential of test substances to induce reverse mutation in the *His* gene of modified *Salmonella typhimurium* strains (Ames et al., 1975; Gatehouse et al., 1994). Mutagenic substances can induce reversion in histidine-deficient strains, which are then able to grow and form colonies in a histidine-limited medium, whereas non-reversed strains cannot grow. A set of six different strains is used in a standard experiment enabling the assessment of various genomic mutations such as base substitutions, additions or deletions of one or more DNA base pairs. The mutagenic activity of the test compound is evaluated through calculation of the mutagenicity ratio, that is, the ratio between the number

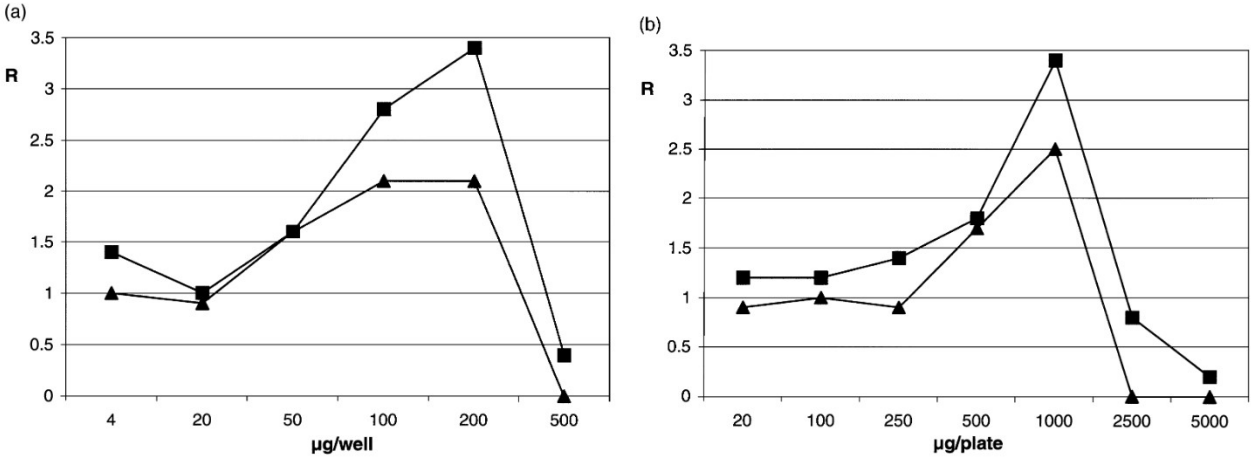

Fig. 1. Dose–response effect observed for compound P5 in the MMT test (panel A) and in the Ames test (panel B). ■, Strain TA1538 in the presence of metabolic activation; ▲, strain TA98 in the presence of metabolic activation.

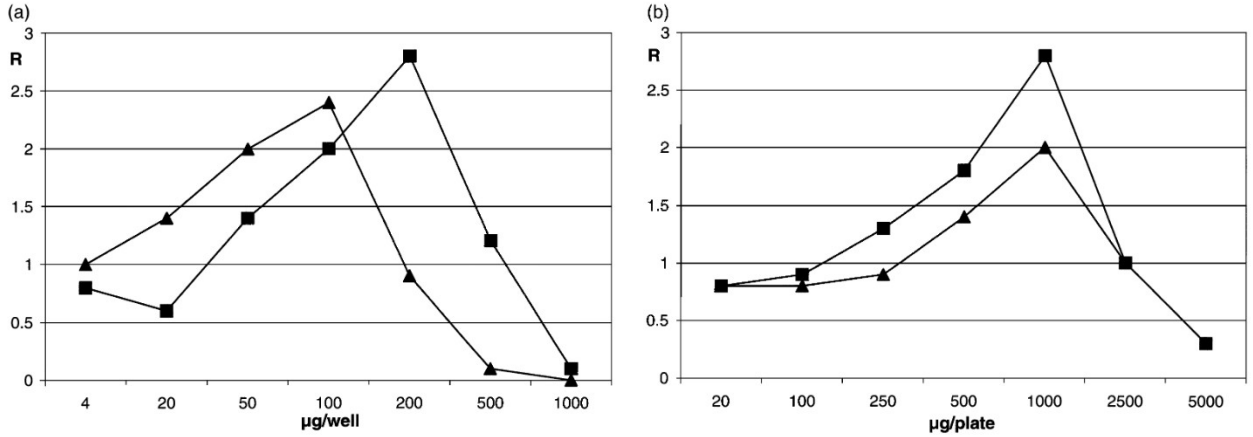

Fig. 2. Dose–response effect observed for compound P6 in the MMT test (panel A) and in the Ames test (panel B). ■, Strain TA1538 in the presence of metabolic activation; ▲, strain TA98 in the presence of metabolic activation.

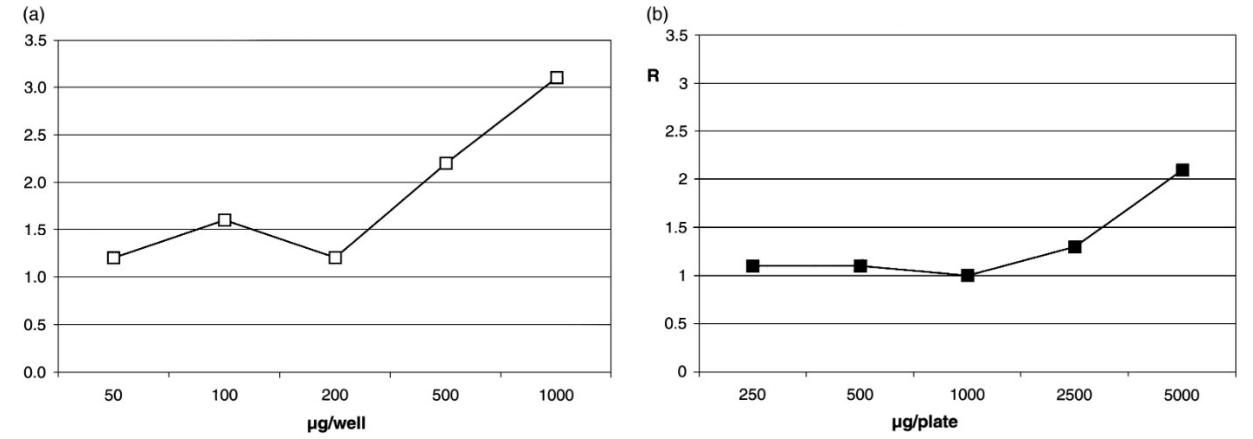

Fig. 3. Dose–response effect observed for the compound P7 in the MMT test (panel A) and in the Ames test (panel B). ■, Strain TA100 in the presence of metabolic activation, pre-incubation protocol.

of revertant colonies scored at a test concentration and the number of revertant colonies scored in the solvent controls (Maron and Ames, 1983). Thus, a quantitative and reliable evaluation of the mutagenic potential of the test compound relies on the possibility to score revertant colonies in the solvent controls.

Several attempts have been made to miniaturise the Ames test on solid agar containing medium (direct

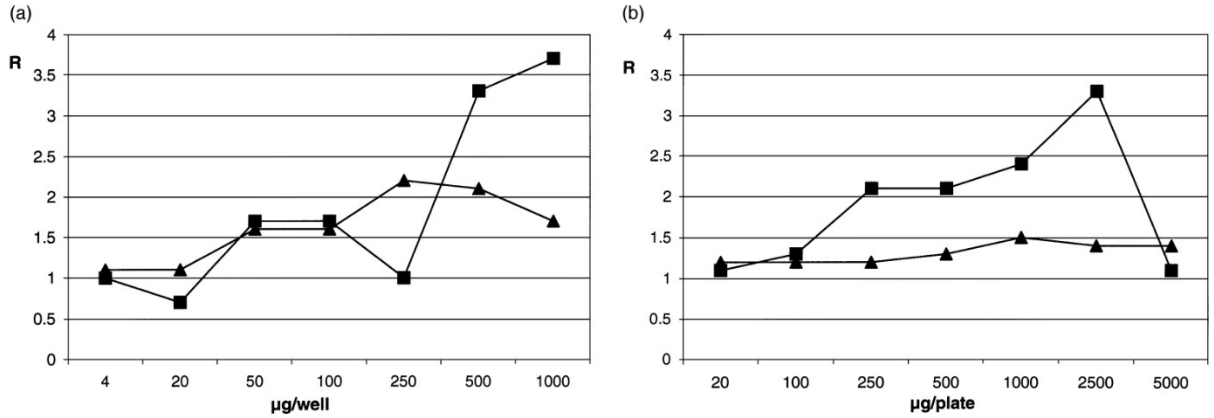

Fig. 4. Dose–response effect observed for the compound P9 in the MMT test (panel A) and in the Ames test (panel B). ■, Strain TA1538 in the presence of metabolic activation, pre-incubation protocol; ▲, strain TA98 in the presence of metabolic activation, pre-incubation protocol.

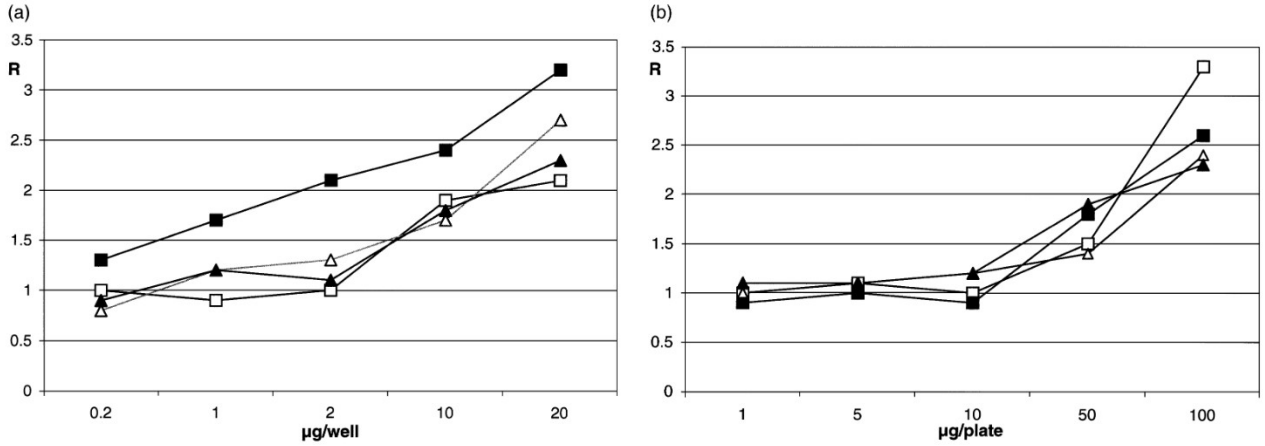

Fig. 5. Dose–response effect observed for MMS in the MMT test (panel A) and in the Ames test (panel B). □, Strain TA100 in the absence of metabolic activation; △, strain TA102 in the absence of metabolic activation; ■, strain TA100 in the presence of metabolic activation; ▲, strain TA102 in the presence of metabolic activation.

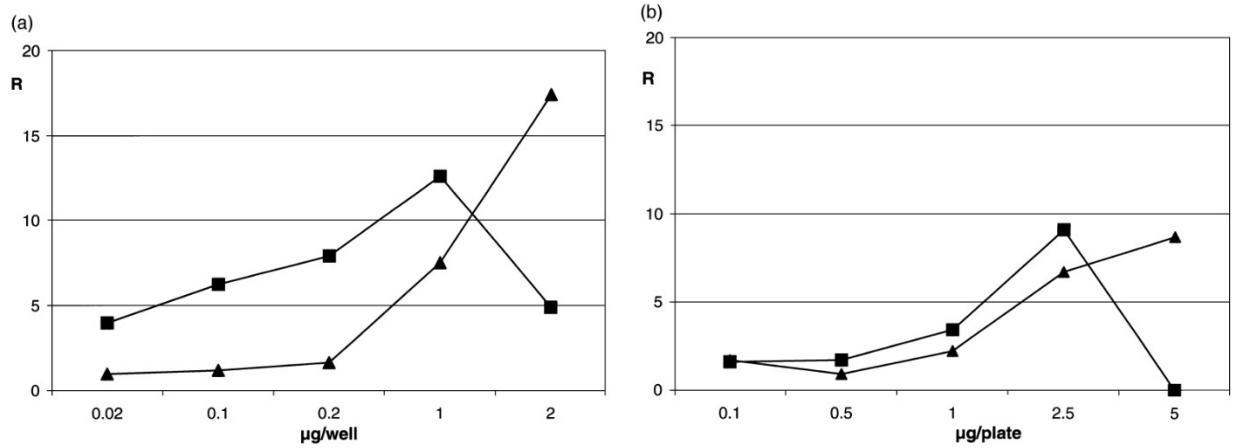

Fig. 6. Dose–response effect observed for 4-NQO in the MMT test (panel A) and in the Ames test (panel B). ■, Strain TA98 in the absence of metabolic activation; ▲, strain TA102 in the absence of metabolic activation.

plating protocol) (Brooks, 1995). The miniscreen assay (Brooks, 1995) was proposed as a miniaturised version of the direct plating protocol of the Ames test and is performed in square 25-well plates. This results in a significant reduction in the quantity of test compound (Brooks, 1995; Burke et al., 1996) needed, but has the disadvantage (i) to require scoring of the colonies under a magnifying glass to obtain quantitative results because

of the greatly reduced size of the revertant colonies (Burke et al., 1996), and more importantly (ii) to be applicable only to the Ames strains exhibiting a high rate of spontaneous revertants. Other miniaturised versions of the modified Ames test were also proposed in 96-well plates and with liquid cultures (Gee et al., 1998). We see two main disadvantages of these liquid methods: (i) first, they do not usually rely on the classical Ames strains (Gee et al., 1994) used in the OECD (1997) protocol, thus the concordance with the regulatory Ames test could be questioned, (ii) second, the use of liquid medium restricts the test substances only to those which are clearly soluble in water or DMSO. The direct plating test on solid agar-based medium allows unsoluble test compounds to be dispersed in the top agar in various solvents [TetraHydroFuran (THF), Isopar L].

Our aim was to develop a miniaturised version of the regulatory Ames test that can be used in a screening assay for mutagenicity, namely: (i) requiring smaller amounts of the test compound; (ii) allowing the evaluation of more compounds in the same experiment; and (iii) being highly predictive for the Ames test. Therefore, we chose to develop a protocol remaining as close as possible to the regular Ames protocol in terms of the *Salmonella* strains and treatment conditions used. The MMT protocol relies strictly on the same protocol as the Ames test but with a reduction by a factor of five of the volume of all reagents used, and the use of six-well plates as culture dishes.

The MMT allows up to three different substances to be tested on the six *Salmonella* strains in a single experiment and results in a significant reduction in the quantity of test compound required. The MMT test requires only 300 mg of the test compound for a complete experiment (six strains, with and without metabolic activation, two independent assays).

Technically, another advantage of the MMT protocol relies on the fact that the scoring of the colonies does not require a magnifying glass. The revertant colonies are only slightly smaller as compared to those observed on 90-mm petri plates, so they can still be easily scored (see Plate 1). Moreover, even if the colonies are slightly smaller in the MMT test, the size and the shape of the true revertant colony in the MMT, and the fact that the cytotoxicity is assessed through an acute observation of the bacterial lawn, enable the spurious non-revertant colonies that could grow under toxic conditions to be easily distinguished from true revertants. Moreover, when toxicity is observed, a second or third test is conducted at lower concentrations of the test compound. The reduction in the number of revertant colonies in the wells of the plate also renders the manual scoring quicker compared to the Ames test. Moreover, the scoring can be done either manually or automatically by image analysis (Noesis SA, Courtaboeuf, France). The six-well plate format (Plate 1) is also very convenient for handling (one plate for each concentration with and without metabolic activation on the same plate, three replicate wells per concentration). The use of these plates, associated with an easy and more uniform pouring of the top agar in the wells (because of their reduced diameter as compared to classical petri dishes) renders feasible a complete automation of the test.

Biologically, the results presented above clearly demonstrate that the MMT protocol allows a quantitative and reliable scoring of the mutagenic activity of a test compound since all strains can be scored, even those that exhibit the lowest spontaneous rate of revertants. A recent paper has been published (Diehl et al., 2000), which presents a miniaturised version of the Ames test close to the MMT presented here. Our data confirm these and even go further, by demonstrating the applicability of this miniaturisation for strains TA1535 and TA1537, which exhibit the lowest revertant rate.

Moreover, this preliminary study on 11 substances demonstrates (i) an excellent concordance between the results of the Ames test and those of the MMT and (ii) a greater sensitivity of the MMT as compared to the Ames test (mutagenic responses can be observed at lower doses).

Up to now, we do not have any explanation for this increased sensitivity (lowering of the threshold of the positive response) observed in the MMT. This phenomenon has to be confirmed by testing more molecules with a higher diversity in chemical structure. For this reason, we are now organising an inter-laboratory study to further support these results with a greater diversity of test compounds. This study will also aim to determine whether the criteria for positivity used in the regulatory Ames test are applicable to the MMT protocol or whether they need to be slightly adapted in order to take into account the observed increase in sensitivity of the MMT protocol.

5. Conclusion

We described here a miniaturised version of the Ames protocol requiring only 300 mg of test compound to evaluate on six strains the mutagenic potential of up to three compounds in the same experiment in a screening assay. This test appears to be fully automatable. The results obtained in a preliminary study on nine in-house test substances and two well-known mutagens confirm the high concordance of the MMT results with regular Ames results.

Thus, this miniaturised protocol presents a clear interest in an industrial screening strategy for genotoxicity testing of raw compounds.

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