Mutation Research 746 (2012) 89–93

Contents lists available at SciVerse ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Short communication

An illusion of hormesis in the Ames test: Statistical significance is not equivalent to biological significance

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ARTICLE INFO

Article history:
Received 13 February 2012
Received in revised form 19 March 2012
Accepted 21 March 2012
Available online 29 March 2012

Keywords:
Hormesis
Salmonella mutagenicity
Ames test
Dose–response
Nonlinear response

ABSTRACT

A recent report (Calabrese et al., Mutat. Res. 726 (2011) 91–97) concluded that an analysis of Ames test mutagenicity data provides evidence of hormesis in mutagenicity dose–response relationships. An examination of the data used in this study and the conclusions regarding hormesis reveal a number of concerns regarding the analyses and possible misinterpretations of the Salmonella data. The claim of hormesis is based on test data from the National Toxicology Program using Salmonella strain TA100. Approximately half of the chemicals regarded as hormetic, and the majority of the specific dose–responses identified as hormetic, were actually nonmutagenic. We conclude that the data provide no evidence of hormetic effects. The Ames test is an excellent measure of bacterial mutagenicity, but the numbers of revertant (mutant) colonies on the plate are the result of a complex interaction between mutagenicity and toxicity, which renders the test inappropriate for demonstrating hormesis in bacterial mutagenicity experiments.

1. Introduction

Dose–response relationships may in principle be linear or curvilinear and can often show thresholds. They may also be biphasic, exhibiting a significant decrease in response at low dose followed by an increase at high dose, or vice versa [1–4]. Hormesis is the term used to describe such a nonlinear curve with significant deviations from the untreated control in opposite directions at high and low doses [1,5]. Hormetic curves may therefore be described as J-shaped when referring to the occurrence of an adverse effect or as an inverted U when describing disruption of a normal function [1–4]. Such responses are often thought to be adaptive, and some consider them to be members of a broad family of biological responses to stress [6]. In the context of mutagenicity, a hormetic response would require a frequency or rate of mutation less than the spontaneous level at low doses, followed by a mutagenic response at higher doses.

The Salmonella/microsome mutagenicity assay, commonly called the Ames test, is undoubtedly the most widely used genetic toxicology test [7,8]. Results from the test are often used to support decisions about whether to proceed with a chemical's development or declare it a potential carcinogen. A positive or negative response in the test for known carcinogens can also influence the risk assessment process for chemicals to which humans are exposed. To establish hormesis for mutagenesis, there first must be clear evidence that the chemical is mutagenic in the specific test. This is critical because nonmutagenic chemicals cannot, by definition, exhibit hormesis for mutagenicity. In addition to showing mutagenicity at the high dose(s), a chemical must have a mutagenic response below the spontaneous level in the low dose range. Demonstrating the latter is inherently problematic, owing to the complex interplay between mutagenicity and toxicity in the assay [9].

Calabrese et al. [10] recently reported that Ames test results in Salmonella strain TA100 provide evidence of hormetic responses. They used statistical approaches to demonstrate that some of the responses that produced a slight increase in absolute numbers of colonies per plate at the highest dose also showed a slight reduction in numbers of colonies per plate at low doses. On this basis they concluded that Ames test data exhibit hormesis. However, there are critical flaws in the design of their study and the interpretation of mutagenicity data, and there is evidence that their findings are not reproducible. Consequently, their study provides no evidence of hormesis. This commentary examines the data used by Calabrese et al. [10] and their conclusions regarding hormesis in the Ames test. Important concerns raised by their report and possible misinterpretations are addressed.

2. Data used for the analyses

The chemicals and the data used by Calabrese et al. [10] were from tests performed and published by the US National Toxicology

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http://dx.doi.org/10.1016/j.mrgentox.2012.03.008

Table 1
Chemicals and activation systems reported to show a hormetic response in Salmonella strain TA100 [10].[a]

Chemical	S9	TA100[b]	Overall[c]	No. tests[d]	Ref.
6-Amino-4-chloro-1-phenol-2-sulfonic acid	R	–	–		[13]
p-Aminophenol	H	–	–	3	[13]
Chlorinated trisodium phosphate	R	?	+w		[13]
Dimethoxane	N	–	+		[11]
Dimethyl hydrogenphosphite	R	+w/?	?	2	[11]
Malonaldehyde Na salt	R	–	–		[11]
Maltol	H	?	+		[11]
Mercuric chloride	H	–	–		[12]
alpha-Naphthyl isothiocyanate	R	?	+		[13]
4-Nitrophthalimide	H	?	+		[13]
p-Phenylenediamine 2HCl	R	?	+		[13]
Pigment green 7	R	–	+		[13]
Succinonitrile	H	–	–	2	[13]
Tetraethyl dithiopyrophosphate	R	–	+		[13]
Triphenylamine	H	–	–	2	[12]
Triphenyl phosphite	H	–	–		[12]

R, induced rat liver S9; H, induced hamster liver S9; N, no S9; +, mutagenic; +w, weakly mutagenic; ?, equivocal response; –, not mutagenic; +w/?, different results in different labs.

[a] Adapted from Table 5 of Calabrese et al. [10].

[b] Published NTP result for the TA100/activation combination reported to show hormesis.

[c] Published result for the chemical taking all Salmonella strain/activation combinations into consideration.

[d] Number of independent tests performed on the chemical. Tests are considered independent if they were conducted in different laboratories, or in the same laboratory at different times under different code numbers.

Program (NTP) [11–13] and/or included in the NTP genetic toxicity database [14]. All data were generated in three laboratories that used the same Salmonella preincubation protocol to test the chemicals under code.

The chemicals identified as producing hormetic responses were from a subset of dose–response data for 825 chemicals tested in Salmonella strains TA97, TA98, and TA100 (approximately 14,500 dose responses). Data on 95 chemicals (107 data sets) were selected as satisfying the initial evaluation criteria [10]. This subset included 61 TA100 data sets from which 16 (26%) satisfied the authors' statistical criteria for a hormetic response at the $p = 0.05$ level (Table 1).

The NTP test protocol and data evaluation criteria are described in detail elsewhere [11–13]. The protocol required a repeat test at least one week later to confirm a positive or negative response in the initial test. A minimum of 5 non-zero concentrations were tested, and the highest test concentration was 10 mg/plate unless limited by toxicity or solubility. Toxicity was identified by a partial-to-complete clearing of the background lawn, a reduced number of revertant (mutant) colonies with increasing doses, or both. All chemicals were tested without metabolic activation (S9) and with liver S9 from rats and hamsters; different S9 concentrations may have been used according to protocol directions. Although the initial test concentrations were at half-log intervals, the laboratory had the option to change the test concentrations or intervals between concentrations for the repeat tests based on the results of the initial test. A number of the chemicals were tested in more than one laboratory or in the same laboratory at different times under different code numbers (Table 1). As stated in the NTP publications [11–13], in most instances only the last of the replicate tests was presented if the results of the replicates were the same with respect to mutagenicity. In addition to the data in the journal publications, all the NTP Salmonella test data, including all test replicates, are publicly available on the NIEHS/NTP web-site [14].

To select chemicals and data sets for the evaluation for hormesis, Calabrese et al. [10] developed a set of a priori "entry criteria" (Table 2). Inspection of the mutagenicity data for the TA100/S9 test conditions that were identified as showing hormesis reveals several areas of concern regarding the published conclusion [10] that the data demonstrate hormesis.

Table 2
Entry criteria used by Calabrese et al. [10].[a]

1. Response at dose #5 is ≥110% of control, and
2. Response at dose #4 is <110% of control;
3. Response at dose #3 is <110% of control;
4. Minimize (solvent) control group variation by an SEM limit of ≤7.5%;
5. Statistical evidence of mutagenicity at dose #5, defined by a response >110% of the control in a one-tailed test at $p \leq 0.10$.

[a] From [10], § 2.1.

3. Concerns and questions addressed

3.1. Evaluation of mutagenicity

If hormesis is defined as a decreased response (i.e., antimutagenic effect) at low test chemical concentrations followed by an increased (mutagenic) response at higher concentrations, it is not possible for a nonmutagenic chemical or a nonmutagenic dose–response relationship to exhibit hormesis.

As can be seen in Table 1, 10 of the 16 chemicals (62.5%) reported to show a statistically significant hormetic response in TA100 are not mutagenic in the particular TA100-S9 combination, and the remaining chemicals produced equivocal or nonreproducible responses. Eight of the chemicals that were not mutagenic or were equivocal in the designated TA100/activation system were mutagenic in other Salmonella strains or with a different metabolic activation system than the one that was identified as showing the hormetic response. Of the 16 chemicals, 7 (44%) are not mutagenic in any Salmonella strain/activation combination.

For the determination of hormesis, a positive response was required at the highest dose tested which, in most cases, was the limit dose of 10 mg/plate[1] or the dose producing toxicity. A 10% increase over the control with a p value ≤0.10 was defined as a positive response [10]. The use of a 10% increase at the highest dose, while an arbitrary and convenient metric, should not be confused with a biologically significant response. We acknowledge that any

[1] The US and international test guidelines for the Salmonella test designate 5 mg/plate as the limit dose for substances that are not toxic. The higher value (10 mg/plate) used by the NTP testing program predated the development of the formal guidelines.

Table 3
Succinonitrile mutagenicity results in TA100 with hamster liver S9.

μg/plate	Lab. 1		Lab. 2	
	10% S9[a]	30% S9[a]	10% S9[b]	30% S9[b]
0	110 ± 11.7	109 ± 5.1	147 ± 7.7	129 ± 5.2
100	121 ± 4.3	110 ± 3.3	131 ± 1.5	120 ± 9.8
333	113 ± 11.3	127 ± 6.9	139 ± 6.7	120 ± 12.3
1000	115 ± 4.8	103 ± 2.4	128 ± 9.3	119 ± 8.7
3333	103 ± 6.4	103 ± 7.1	133 ± 5.5	135 ± 8.6
10,000	105 ± 8.1	122 ± 3.2	119 ± 7.2	127 ± 2.8

Mutagenicity responses are the means of triplicate plates ± SEM.
[a] Data from [14].
[b] Data from [13].

distinction between mutagenic and nonmutagenic responses in the test is subjective. However, requiring only a 10% increase, often at the highest testable toxic or subtoxic dose, is insufficiently conservative. Such a response would not be considered positive in any properly designed mutagenicity testing program.

Criteria for a positive response in the Ames assay have been much discussed in the genetic toxicology literature. The most common criterion is a dose-related increase in revertants per plate achieving an increase greater than twofold (in the absence of a repeat test). While arbitrary, this rule of thumb has yielded conclusions consistent with other widely used criteria, such as a reproducible dose-dependent increase in colonies per plate, even if less than twofold, or a reproducible, statistically significant increase in numbers of colonies per plate as part of a dose–response. The papers from the NTP study [11–13] identify the criteria that were used by the labs that performed the assays. The analysis by Calabrese et al. [10] did not follow these conventional criteria or other approaches that correspond to norms in the field.

An example of a data set that was judged as being hormetic [10] but shows a minimal, if any, increase in revertants over background is that of the nonmutagen succinonitrile, which was tested in two laboratories using two S9 concentrations (Table 3). Of the four TA100 data sets for this chemical, only one (Lab 1 with 30% S9) shows any increase over the control at the higher doses, from 109 ± 5.1 to 122 ± 3.2 at the limit dose of 10 mg/plate (an 11% increase). An increase of this magnitude would not be considered evidence of mutagenicity in any testing program. It is also worth noting that the complete dose–response for this test does not show the U-shaped curve that defines hormesis.

It is a concern when chemicals and individual tests that have been shown to be nonmutagenic in properly designed and interpreted assays are considered to show mutagenicity and hormesis. This issue goes directly to the definition of hormesis, which requires that the substance exhibit a toxic (in this case, mutagenic) response at higher doses than those that are considered protective.

3.2. Reduced numbers of revertants at low dose

By definition, hormesis requires a decreased (antimutagenic) response at doses below those that produce a mutagenic response. This was not always achieved in the tests identified as hormetic.

The succinonitrile example (Table 3) used to illustrate an incorrect claim of mutagenicity also illustrates this problem. Another example is the response to pigment green 7 (Table 4). This chemical was identified as showing hormesis in TA100 with rat liver S9 [10]. A dose-related response was observed in TA100, but the chemical was not considered mutagenic in this strain [13]. This TA100 test was not reproduced by the laboratory, but pigment green 7 was judged mutagenic based on its responses in Salmonella strain TA98 with rat and hamster S9 [13]. With respect to the analysis of hormesis, it is striking that there is no convincing decrease in numbers of revertants per plate at low doses in strain TA100 (Table 4).

Table 4
Pigment green 7 mutagenicity results in TA100 with rat liver S9.

μg/plate	30% S9[a]
0	138 ± 5.0
100	131 ± 9.1
333	138 ± 14.7
1000	151 ± 6.2
3333	145 ± 6.6
10,000	157 ± 9.0

Mutagenicity responses are the means of triplicate plates ± SEM.
[a] Data from [13].

Although the 100 μg response (131 ± 9.1) is below the control value of 138 ± 5.0, the magnitudes of the SEM values are consistent with this being the result of the normal, stochastic fluctuation around the mean.

3.3. Reproducibility

If hormesis exists, the response should be reproducible within and among laboratories testing the same chemicals using identical protocols and dose levels. Reproducibility is critical for demonstrating the validity of a scientific result, and most of the NTP tests were replicated by the testing laboratories before submitting the data. In the majority of cases, these replicates used the same doses and/or dose ranges as the initial test. Although most of the tests identified as hormetic were reproduced by the testing laboratory, and occasionally by a second laboratory (Table 1) using the same protocol and same doses or dose range, there was little or no reproducibility of the response pattern identified as hormetic.

The mutagen *p*-phenylenediamine (Table 5), which was reported to show hormesis in strain TA100 with rat liver S9, is one such example, in that the response was not consistent across replicates with respect to evidence for hormesis. The overall determination of mutagenicity for this chemical was based on the positive responses in strain TA100 with hamster S9 and in TA98 with rat and hamster S9. Although the initial run, with all doses showing increases over the control, was judged mutagenic by the NTP [14], the second and third replicate experiments were judged negative or equivocal because all dose levels showed decreases from the control in Run 2, and only the high dose was increased in Run 3 [13,14]. The only response that could be considered as showing a hormetic pattern was in Run 3 – one of three identical, replicate experiments performed in this laboratory; i.e., the hormetic-type response was not reproducible.

The evidence for hormesis in the Ames test [10] also suffers from a lack of reproducibility between labs. The nonmutagen *p*-aminophenol was tested as three independent coded samples in two laboratories (Table 6). It was reported to show hormesis based on the response in TA100 with hamster liver S9 [10]. The specific data set(s) that were considered hormetic were not identified, but it appears as though the data for 30% S9 from Lab 1(a and b) fit

Table 5
p-Phenylenediamine 2HCl mutagenicity results in TA100 with rat liver S9.

μg/plate	30% S9: Run 1[a]	30% S9: Run 2[a]	30% S9: Run 3[b]
0	132 ± 5.2	111 ± 2.7	118 ± 0.3
100	138 ± 1.5 (c)	97 ± 5.5	101 ± 4.0
250	150 ± 8.9	94 ± 5.9	106 ± 4.3
1000	134 ± 3.2	98 ± 4.7	109 ± 4.1
2500	173 ± 7.2	104 ± 4.5	115 ± 0.7
10,000	417 ± 16.2 (p)	97 ± 0.3 (p)(s)	155 ± 21.8 (p)(s)

Mutagenicity responses are the means of triplicate plates ± SEM.
c, contamination on plates; p, precipitate on plates; s, slight toxicity.
[a] Data from [14].
[b] Data from [13].

Table 6
p-Aminophenol mutagenicity results in TA100 with hamster liver S9.

μg/plate	Lab. 1a		Lab. 1b		Lab. 2	
	10% S9[a]	30% S9[a]	10% S9[a]	30% S9[a]	10% S9[a]	30% S9[a]
0	126 ± 7.1	138 ± 5.4	153 ± 9.4	135 ± 3.0	106 ± 3.8	114 ± 10.4
10				110 ± 4.7		
33	141 ± 2.5	129 ± 7.2	143 ± 0.3	128 ± 9.6	107 ± 4.0	116 ± 10.7
100	149 ± 13.4	102 ± 3.2	128 ± 6.8	129 ± 10.1	106 ± 0.9	110 ± 3.3
333	153 ± 1.9	139 ± 10.4	154 ± 1.9	146 ± 13.0	111 ± 3.5	132 ± 2.6
1000	70 ± 5.3	155 ± 6.3	103 ± 2.3	165 ± 16.8	113 ± 3.4	115 ± 7.8
1666		91 ± 14.3				
2000					110 ± 5.8	62 ± 2.9
3333	6 ± 2.2 (s)		0 (s)			

The same test chemical batch was used in all three tests [13]. Lab 1a and 1b represent two independent tests with differently coded samples in the same laboratory. Mutagenicity responses are the means of triplicate plates ± SEM.
s, slight toxicity
[a] Data from [13].

the entry criteria. The response, however, was not found by Lab 2, and it was not seen with 10% S9. Given the closeness of the protocols among these independent tests, it would be expected that a true hormetic response would be seen consistently in these data sets.

If hormesis were present and if it were a function of the test chemical and the test endpoint, it should manifest itself in all the replicate tests from the same lab and in tests from different labs that used the same dose range and testing conditions. The requirement of reproducibility was not met by all the experiments used as evidence of hormesis.

3.4. Suitability of the Ames test for studying hormesis

Decreases in colony count in the Ames test do not simply reflect a decrease in mutation rate or mutation frequency. The revertant colonies recovered in the Salmonella test result from a competition between the toxic and the mutagenic effects of the test substance [9]. This interplay between toxicity and mutagenicity may complicate data interpretation, but it does not prevent the unequivocal measurement of mutagenicity. A mutagenic chemical can sometimes produce an increase in colonies even when there are fewer viable cells on the plate.

For this reason, the standard Ames test is not suitable for the measurement of hormesis. The Ames test does not measure a mutation rate or even a revertant frequency per viable cell. Instead, it measures revertant colonies per plate with no corresponding measurement of the size of the cell population at risk. This is not a concern if there are substantial increases in numbers of revertant colonies and the only issue is whether the test substance is mutagenic. Numbers of colonies per plate serve as a surrogate for mutation frequency in this case because no other common biological factor will increase the colony count if appropriate precautions are taken to monitor for extreme toxicity, avoid contaminants, and properly control the histidine concentration.

Because most toxicity mechanisms do not lead to mutation, it is possible for toxicity to occur at lower doses than mutagenicity. If a chemical has no effect at the lowest dose, exhibits modest toxicity at intermediate doses, and is mutagenic at higher doses, the resulting dose response could appear to be hormetic. Toxicity at intermediate doses could lead to a modest decrease in surviving spontaneous revertants, which would mimic a hormetic response, but it would not represent an actual decline in the frequency of mutations per viable cell. Growth inhibition could also lead to a reduction in colonies per plate. These effects can be subtle at lower doses, so that modest decreases in colony count, by themselves, support no clear conclusion as to a mechanism. Toxicity and growth inhibition are not equivalent to hormesis for mutagenicity. Therefore, although the Ames assay is an excellent test for bacterial mutagenicity, it is less suitable for an evaluation of hormesis.

4. Discussion

Calabrese et al. [10] used a statistical approach to show that some Ames test responses with a slight increase in absolute numbers of colonies per plate at the highest dose also show slight reductions in numbers of colonies per plate at lower doses. This statistical finding for isolated tests from a few chemicals does not translate into a demonstration of hormesis.

To establish hormesis in a mutagenicity test, there first must be clear evidence that the chemical is mutagenic in the test. Criteria for positive responses in the Ames assay have been discussed in the literature for more than 30 years. Calabrese et al. [10] praise the quality of the data and the expertise of the laboratories that performed the assays but do not follow the criteria for mutagenicity that the laboratories producing the data at issue had used [11–14]. Instead, a nonreproducible 10% increase in colonies per plate at a single dose was chosen as evidence that a response is positive. This was reinforced by an ad hoc statistical procedure (a one-tailed t test with a p value of 0.10) that was not well defined or justified. This is a criterion that has never before been seen in our combined experience. A new alternative criterion may be fine, but it would need to be justified. If a 10% increase in colony count only at the highest test dose is taken as realistic evidence for a mutagenic response in the Ames assay, one could go back through past data and find many new putative mutagens that have been declared nonmutagenic over the past 40 years. If the mutagenicity of a substance is doubtful, there can be no unequivocal demonstration of hormesis. The majority of the chemicals reported to be hormetic by Calabrese et al. [10] were judged nonmutagenic by the test laboratories [11–14]. These judgments should not be negated to enable a judgment of hormesis.

In reporting that 16 chemical/TA100/activation tests show hormesis, Calabrese et al. [10] provided no information on whether the putatively hormetic responses were reproducible and consistent with other tests of the same chemicals. The examples that we have presented indicate that such reproducibility was lacking.

Even if the analysis were restricted to bona fide mutagens, mechanistic considerations argue that the Ames test does not lend itself to an evaluation of hormesis. The revertant colony counts in the Ames test derive from a complex interaction between mutagenicity and toxicity [9]. As a consequence, increases in the number of colonies can unequivocally indicate mutagenicity, but decreases in the number of colonies at low or intermediate doses leave the causative mechanism unknown. Inasmuch as a decrease may reflect growth inhibition or other manifestations of toxicity, it cannot be ascribed to hormesis in the absence of mechanistic

information. To show a hormetic response, there must be consistency with the biology of the assay, consistency across exposure regimens, and reproducibility among replicate tests.

An issue that should be considered when the report of a biological effect rests heavily on statistical considerations is the extent to which a purely statistical argument should override conclusions that are reached by observation of the data. It is not enough to look at the calculated *p* value for the chosen statistic when attempting to determine whether a biological effect is present. One must also critically evaluate the actual data in the context of the assay to determine whether the statistical conclusion is reflected in the data or whether the data appear to contradict the statistical conclusion. Evaluation of the results from replicate experiments is essential because the hallmark of a true scientific finding is its reproducibility. The report of hormesis among the chemicals identified by Calabrese et al. [10] does not always meet the criterion of reproducibility, and it inappropriately uses responses from tests that showed nonmutagenicity or questionable mutagenic responses. These considerations call into question the biological relevance of these statistically based conclusions.

A claim of hormesis has important implications because of its bearing on the fundamental nature of dose–response relationships and because it may be cited as relevant to risk assessment for mutagens and carcinogens [3,10,15]. In previous studies, Calabrese and colleagues have presented convincing evidence that the hormesis phenomenon is real for various biological endpoints [16,17]. The presence of chemically induced biphasic responses in toxicology is well established and supported by biological considerations [2,16,18]. Thus, the existence of low-dose, nonlinear responses is not at issue. However, in the case of the reported hormesis in the Ames test [10], it is likely that factors other than hormesis affected the outcome of the analysis. These include inappropriate criteria for determining mutagenicity, hyper-Poisson variability in the TA100 colony counts [9] (which was not addressed by the statistics used), the fact that there is more than one possible cause for decreases in numbers of colonies, and random variation in the absence of evidence of reproducibility. The data and interpretations used as evidence of hormesis in the Salmonella mutagenicity test do not stand up to scrutiny.

5. Conclusion

Calabrese et al. [10] have shown through a statistical analysis of Ames test data that there is a tendency toward slight reductions in colony counts at low doses when there is a slight increase in colony count at the highest dose. This may be of little importance, and it is a long way from demonstrating hormesis for mutagenicity. Although hormesis may occur for mutagenicity, claiming that it is evidenced on the basis of this data set and analysis can be seriously misleading.

Conflicts of interest

None.

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