Mutation Research, 113 (1983) 13–19
Elsevier Biomedical Press

The effect of different genetic properties of *Salmonella typhimurium* on the determination of stabilities and mutagenic concentrations of chemical carcinogens using the diffusion bioassay

Tamara E. Awerbuch *,** and Anthony J. Sinskey

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, MA 02139 (U.S.A.)

Summary

The mathematical model used to calculate half-life and mutagenic concentrations of chemical carcinogens from the diffusion bioassay does not include any terms related to the nature of the microorganism used in the assay (Awerbuch et al., 1979; Awerbuch and Sinskey, 1980). In this work we tested the model with different strains of *Salmonella typhimurium*. These strains are auxotrophs for histidine and are sensitive to base-pair substitution.

The half-life ($\tau_{1/2}$) of *N*-methyl*N'*-nitro-*N*-nitrosoguanidine (NG) was calculated by the diffusion assay, using strains *hisG46*, TA1950, TA1535 and TA100 as the bacterial indicators. For all strains $\tau_{1/2}$ equalled 2.2 h; strain sensitivity for detecting threshold mutagenic concentrations of NG was essentially the same, except that *hisG46* was slightly more sensitive.

A quantitative model of an experiment for detecting the biological activity of a chemical was presented previously (Awerbuch et al., 1979). In that experiment, a droplet of the chemical was placed at the center of a petri dish containing a bacterial lawn in an agar gel.

The main results of the mathematical treatment of the model were: (a) an equation to determine the half-life of the chemical and (b) a functional relationship between the radius at a certain point of the dish and the concentration of the chemical at this point.

* Present address: Memorial Sloan Kettering Cancer Center, 1275 York Ave., Box 469, New York, NY 10021 (U.S.A.).

** Author to whom all correspondence should be sent.

0165-1161/83/0000–0000/$03.00

These equations were successfully used to determine the stability of mutagens in the environment as well as the threshold mutagenic concentrations of these chemicals (Awerbuch and Sinskey, 1980). We performed a series of diffusion experiments with different mutagens, some of which needed metabolic activation, and measured the external radii of the mutagenic rings which formed after an incubation period. The values of the measured radii were then inserted in the proper equations.

All these experiments were performed with *Salmonella typhimurium* TA1535, which has a histidine requirement and is used to detect mutations through base-pair substitution; it has a deletion through the *uvrB* region of the chromosome, which eliminates the excision-repair system for DNA, and an *rfa* (deep-rough) mutation resulting in a deficient lipopolysaccharide that coats the bacterium and makes it more permeable to the mutagen (Ames et al., 1973b). This strain, derived from *hisG46*, provides a greater sensitivity for the detection of mutagens.

The present study tests the universality of the model for the diffusion bioassay with respect to the bacterial strain.

The half-life of the mutagen is obtained from the slope of the linear relationship between the logarithm of the initial concentration of the mutagen at the center of the dish and the size of the mutagenic zone:

$$\ln c_0 = \frac{r_{\text{mut}}}{\sqrt{D\tau}} + \text{constant} \qquad \text{(Awerbuch et al., 1979)}$$

We obtained the slope by using different initial concentrations (c_0) and then plotting their logarithms against the respective resulting mutagenic zones (r_{mut}). D, the diffusion coefficient, can be easily determined (Awerbuch and Sinskey, 1980); thus one can calculate $\tau_{1/2}$, the half-life of the mutagen.

We wanted to see whether the values for $\tau_{1/2}$, obtained by calculations from the diffusion bioassay using *S. typhimurium* TA1535 and confirmed by spectrophotometrical studies, are independent of the strain used as the bacterial indicator.

We also wanted to see whether *S. typhimurium* strains with different permeabilities and repair capabilities vary in their sensitivity to threshold mutagenic concentrations. For this purpose we performed diffusion experiments with *S. typhimurium* strains derived from *hisG46* and selected for various genetic properties. All strains used contained the histidine mutation which is a missense one and is reverted well by mutagens that cause base-pair substitution (Ames et al., 1973b). The relevant characteristics of the strains used in the assay are described in Table 1.

Materials and methods

The experimental methods are essentially the same as those described by Awerbuch and Sinskey (1980).

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) was purchased from Mann Research Labs. *S. typhimurium* strain TA1535 was provided by Dr. P. Kraft and TA100 by Dr. A. Stark, both from the Department of Nutrition and Food Science, M.I.T.; strains *hisG46* and TA1950 were given by Dr. G. Walker from the Department of Biology, M.I.T.

TABLE 1

GENOTYPE OF THE STRAINS USED FOR TESTING MUTAGENS

Strain	LPS (lipopolysaccharide)	DNA repair	R factor plasmid
hisG46 [a]	+	+	−
TA1950	+	Δ*uvrB*	−
TA1535 [a]	*rfa*	Δ*uvrB*	−
TA100 [b]	*rfa*	Δ*uvrB*	+

All strains were originally derived from *S. typhimurium hisG46* (Ames et al., 1973b). + indicates a wild-type gene. Δ indicates a deletion. The mutagen tested for its $\tau_{1/2}$ and threshold concentration was NG.

[a] Ames et al. (1973b).

[b] McCann et al. (1975).

The preparation of the agar plates, the spectrophotometrical measurements and the diffusion bioassay were as previously described (Awerbuch and Sinskey, 1980). The concentrations of the 0.025 ml NG placed on the disc at the center of the dish were 1000, 100 and 10 μg/ml.

Results

For different initial concentrations of NG, different ring sizes of mutants were obtained. Table 2 lists the r_{mut} for 3 strains that differ in their repair capabilities and in their permeability to the mutagen.

All of the strains give straight lines when $\ln c_0$ is plotted against r_{mut} (Fig. 1). Strains TA1535 and TA1950, different only in that TA1950 has an intact LPS, give essentially the same values for r_{mut} when the same concentrations of NG are placed at the center of the dish. This finding indicates that the bacterial coat has no effect on the sensitivity of the test.

When strain *hisG46* — which has no deletion through the *uvrB* region of its genome and has an intact LPS — is used as the bacterial indicator, we get a line parallel to the one obtained with TA1950 and TA1535, but with higher values for r_{mut}. The *hisG46* is more sensitive for detecting threshold concentrations of the chemical; but since it has the same slope as the other strains, it gives practically the same calculated value for $\tau_{1/2}$, i.e., $\tau_{1/2} = 2.24$ h as calculated from the line obtained with strains TA1535 and TA1950; $\tau_{1/2} = 2.16$ h as calculated from the line obtained with strain *hisG46*. A value of $D = 7.2 \times 10^{-6}$ cm^2/sec was used for the calculation (Awerbuch and Sinskey, 1980).

In a separate set of experiments, we compared strains TA100 and TA1535 to see whether the plasmid pKM101 contained in TA100 would affect the slope of $\ln c_0$ versus r_{mut} and thus give a different value for $\tau_{1/2}$. The values of r_{mut} corresponding to different values of c_0 are given in Table 3.

TABLE 2

SIZE OF THE EXTERNAL RADIUS OF THE MUTANTS' RING FOR *hisG46*, TA1950 AND TA1535

c_0 (μg/ml)	r_{mut} (cm)		
	hisG46	TA1950	TA1535
1000	2.2 ±0.04	1.96±0.06	1.86±0.05
100	1.62±0.04	1.28±0.07	1.24±0.06
10	0.99±0.03	0.53±0.05	0.59±0.09
0	0	0	0

0.025 ml of different initial concentration of NG were placed at the center of the dish containing a lawn of *S. typhimurium*. The radii were measured for different strains after 2.5 days of incubation at 37°C. r_{mut} values are means of 6 measurements±standard errors of the mean. The radius of the petri dish2.5 cm.

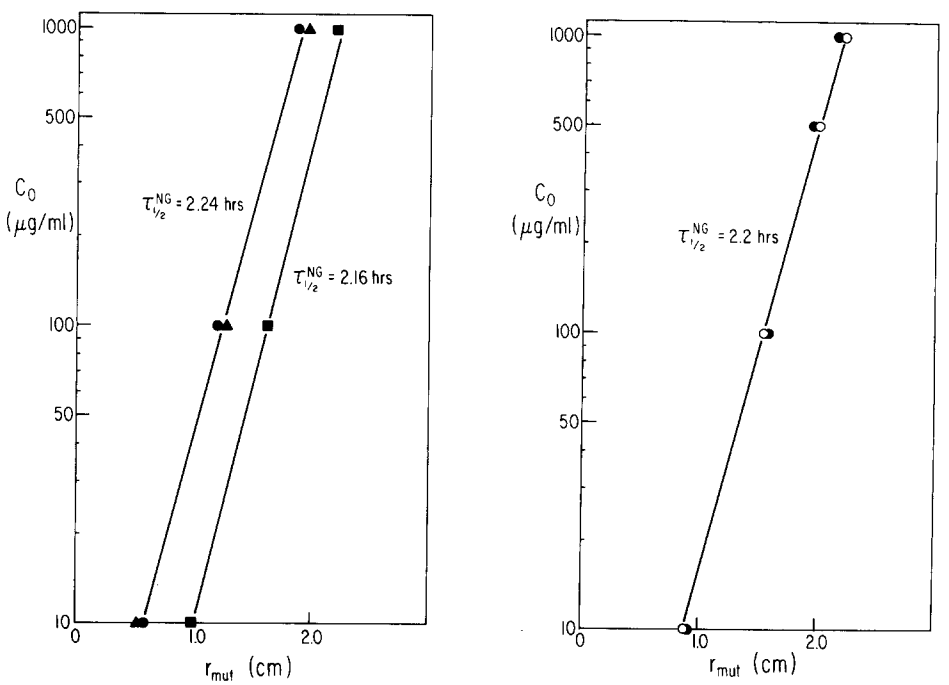

Fig. 1. The natural logarithm of the initial dose c_0 of NG placed on a 6.3-mm filter paper at the center of the petri dish containing lawns of different *S. typhimurium* strains versus the size of the radius of the mutagenic zone r_{mut} (ln c_0 vs. r_{mut}). ●, TA1535; ▲, TA1950; ■, *hisG46*. The radii were measured after 2.5 days of incubation at 37°C.

Fig. 2. The natural logarithm of the initial dose of NG placed on a 6.3-mm filter paper at the center of the petri dish containing lawns of *S. typhimurium* TA1535 (○), and TA100 (●), versus the size of the radius of the mutagenic zone r_{mut} (ln c_0 vs. r_{mut}). The radii were measured after 2.5 days of incubation in a 5% CO_2 incubation at 37°C.

TABLE 3

SIZE OF THE EXTERNAL RADIUS OF MUTANTS' RING FOR TA100 AND TA1535

c_0 (μg/ml)	r_{mut} (cm)	
	TA1535	TA100
1000	2.22±0.05	2.16±0.05
500	1.98±0.05	1.95±0.02
100	1.55±0.05	1.57±0.06
10	0.88±0.05	0.92±0.05
0	0	0

0.025 ml of different initial concentrations of NG were placed at the center of a dish containing a lawn of *S. typhimurium*. The radii were measured after 2.5 days of incubation in a 5% CO_2 incubator at 37°C. r_{mut} values are means of 6 measurements ± standard errors of the mean. The radius of the petri dish = 2.5 cm.

When we plot the values of ln c_0 against r_{mut} (Fig. 2), we get practically the same line for both strains. Thus in this assay, TA1535 and TA100 give the same value for $\tau_{1/2}$ (2.2 h) and are equally sensitive for detecting threshold concentrations for the mutagenic activity of chemical carcinogens.

Although the $\tau_{1/2}$ values derived from 2 sets of experiments using T1535 as the bacterial indicator (Tables 2 and 3) are the same, the r_{mut} values are somewhat different. The values are higher in Table 3; this is probably due to differences in experimental conditions; the plates were incubated in different incubators with different gas mixtures (see captions to tables). $\tau_{1/2}$ is the same in both cases because it is determined by the slope of the line ln c_0 vs. r_{mut} and not by the absolute values of r_{mut}.

Discussion and conclusions

The equation

$$\ln c_0 = \frac{r_{mut}}{\sqrt{D\tau}} + \text{constant}$$

which enables us to calculate the decay time of mutagens from a diffusion bioassay does not include any term regarding the nature of the strain used as the bacterial indicator, thus implying that the half-life of the mutagen obtained using this calculation is of a general nature. In previous work the half-life of NG measured independently by spectrophotometry was 2.25 h (Awerbuch and Sinskey, 1980). This finding agrees with the values calculated from the diffusion experiment: i.e., $\tau_{1/2}$ = 2.24 h in cases where strains TA1950, TA1535 and TA100 were applied for the formation of the lawn; $\tau_{1/2}$ = 2.16 h when *hisG46* was used. Since strains TA1950 and TA1535 differ only in that TA1535 has an *rfa* mutation, which makes the bacterium more permeable to the mutagen, we can conclude that this trait does not

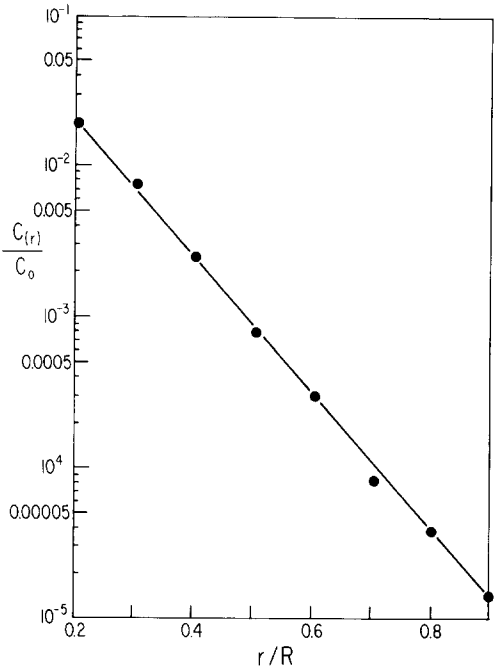

Fig. 3. Logarithmic plot of the time average concentration of NG as a fraction of the initial concentration versus the relative distance from the center, as calculated from equation of previous work (Awerbuch et al., 1979).

affect the decay rate of the mutagen in the system; nor is the stability of the mutagen affected by the deletion of the *uvrB* region of the chromosome, as evidenced in the equal values for $\tau_{1/2}$ obtained with *hisG46* and TA1535.

Nevertheless one has to point out that for 90% confidence intervals on the difference between 2 slopes derived from experiments with different bacterial indicators, the difference can be as large as 20%

The R factor plasmid (pKM101) in TA100, the only characteristic which genetically distinguishes the strain from TA1535, also did not influence the sensitivity of the test for determining threshold chemical concentrations for mutagenicity; the same values for r_{mut} were obtained for the same initial concentrations of mutagens placed at the center of the dish. This result was unexpected since strain TA100 is much more sensitive than TA1535 for NG and for other mutagens in a standard plate assay developed by Ames et al. (1973a).

Larger sizes of r_{mut} were obtained with strain *hisG46* than with the other strains in the same set of experiments. The actual values for the threshold are obtained by using Fig. 3, which presents the change of the mutagen concentration in the dish as a function of the distance from the center. The derivation of this figure and the way it should be used for the calculation of mutagenic concentrations of chemical carcinogens are given elsewhere (Awerbuch et al., 1979; Awerbuch and Sinskey, 1980). When the values of r_{mut} obtained with 10 μg/ml of 0.025 ml of NG on a 6.3-mm

filter (Table 2) are used to calculate the threshold mutagenic concentration from Fig. 3, we find that $C_{mut} = 5.5 \times 10^{-3}$ μg/ml when *hisG46* is used for the lawn; when the other strains are used $C_{mut} = 3.4 \times 10^{-2}$ μg/ml. This greater sensitivity of *hisG46* to NG agrees with the findings obtained with the usual standard plate assay, that *hisG46* is more sensitive than TA1535 to low molecular weight alkylating agents (Ames et al., 1973a); however, the difference in C_{mut} obtained with *hisG46* and the other strains is not considered significant in view of the sensitivity of the diffusion assay, which can detect differences between values with an accuracy that lies within an order of magnitude (Awerbuch and Sinskey, 1980).

Acknowledgements

Part of this work was supported by a grant from the National Institute of Health (NIH 5P0/ESOD597).

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