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Plate Diffusion Assay as a Rapid Method for Dosimetry of Mutagens

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This paper presents a method for determining mutagenic concentrations of chemicals by using an agar diffusion assay. The method is based on the linear relationship between the amount of chemical placed at the center of the dish and the radius of the mutagenic zone. A brief theoretical discussion and experimental data confirming this relationship are given. Alkylating agents and mycotoxins were used to test the system. This method can be used to follow up decreased mutagenic potencies of solutions of unstable mutagens and to follow the production of mutagenic substances throughout fermentation.

Agar diffusion methods for estimating growth inhibitory activities of antibiotics were in use long before theoretical description of the rules governing the appearance of growth inhibition zones existed. They are based on the observed relationship between the amount of antibiotic placed in a reservoir on solid medium and the diameter of the growth inhibition zone (3).

Quantitative antibiotic assay is carried out by comparing growth inhibition zone diameters of the tested compound with those formed by known amounts of the antibiotic. A dose-response standard curve can be constructed by plotting the logarithm of the dose applied against the square of the zone diameter:

$$\ln c_0 \alpha d^2 \quad (1)$$

or, in some cases, against the zone diameter only:

$$\ln c_0 \alpha d \quad (2)$$

as an approximation of the former (6), where c_0 = initial antibiotic concentration and d = zone diameter. Samples of the antibiotic of unknown concentration are diluted serially, and the developing growth inhibition zones are measured and used to read the antibiotic concentration off the standard curve. Standard curves and unknown samples are exposed to the same experimental conditions (e.g., medium, temperature, plate thickness, sample volume, seeding density of indicator organism, exposure time) to minimize nonspecific variations in zone diameters.

We propose to apply similar concepts to estimate the potency of mutagen samples of unknown concentrations using the Ames spot test (1), which until now has been employed for qualitative purposes. Quantitative spot tests for mutagens can be carried out in practice if it is proven that indeed there is a functional relationship between the radii of mutant zones and initial concentrations of the diffusing mutagen. Such a relationship would allow the construction of dose-response standard curves.

Theoretical considerations. A paper disk containing mutagen solution is placed at the center of a petri dish containing a uniform suspension of a tester strain embedded in agar. The mutagen diffuses outward, forming a concentration gradient that varies with the position on the plate and with time. The gradient thus established causes mutants to appear at sites where the concentration reaches mutagenic levels and yet is not sufficient to cause massive cell death. The model and its mathematical treatment are published in detail elsewhere (1a), and a summary is presented here.

The basic assumption is that the appearance of mutants is governed exclusively by the local-temporal concentration $c(r,t)$ of the diffusing mutagens, which obeys a two-dimensional diffusion equation:

$$\frac{\partial c}{\partial t} = D\left(\frac{\partial^2 c}{\partial r^2} + \frac{1}{r}\frac{\partial c}{\partial r}\right) - \frac{c}{\tau} \quad (3)$$

where r = the distance from the center of the petri dish to the mutant ring's outer boundary; ∂ = partial derivative; t = time (from the moment of mutagen application); D = the diffusion coefficient of the mutagen in the agar gel; and τ = the half-life time of the mutagen divided by ln2 (i.e., decay time). Other physicochemical param-

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eters such as pH and temperature are taken into account only insofar as they affect $c(r,t)$ through the quantities D and τ. Other investigators have used a similar equation to describe theoretically the formation of inhibition zones in response to antibiotics (3). In solving the equation, they assumed that although bacteria, at any point on the plate, were exposed to a gradient of concentration that passed with time, they were actually affected by only one concentration. If this level was higher than a critical threshold (typical of the indicator used), growth was inhibited; otherwise most cells survived. Thus, taking this assumption into account in solving the differential equation, the antibiotic concentration placed in the reservoir was proportional to the square of the growth inhibition radius (equation 1) (3). However, in mutagenicity testing, we assume that the probability of a mutagen interacting with the bacterial deoxyribonucleic acid and hence producing a mutant colony does not depend solely on a single critical concentration of the mutagen in question, but also on the period of time during which the bacteria are exposed to the mutagen.

The combined effects of time and concentration are taken into account in this case by using the integrated concentration over time (i.e., exposure) as the criterion for the appearance or nonappearance of mutant colonies at certain sites of the petri dish. Calculations of exposures are carried out with the aid of a computer. The theoretical considerations and the mathematical treatment are described in detail elsewhere (1a). For the practical purposes of constructing a dose-response curve, it is sufficient to know that the mathematical treatment based on the assumption stated above renders the functional relationship:

$$\ln c_0 \alpha r \quad (4)$$

for those mutagens with a decay time (τ) shorter than $R^2/4D$, where R = the radius of the petri dish and D = the diffusion coefficient of the mutagen in question.

In this work, we show that development of mutant rings induced by several known chemical mutagens/carcinogens and several naturally occurring mutagenic mycotoxins obeys the mathematical functions based on the above-mentioned theoretical considerations. We also show that, as a result, quantitative dose-response standard curves can be obtained from the Ames spot test, thus making the test a means for quantitatively estimating mutagens in solution. Some useful applications of this system are also described.

MATERIALS AND METHODS

Bacterial strains. *Salmonella typhimurium* TA1535 was obtained from B. N. Ames (University of California, Berkeley). Strain TM677 (a prototrophic revertant of strain TA1535, into which the plasmid pKM101 was inserted) was a gift from W. G. Thilly (Massachusetts Institute of Technology, Cambridge).

Bacteriological procedures and mutagenesis testing. Bacteriological procedures and mutagenesis testing of strain TA1535 in the histidine-reversion assay (spot test) were carried out according to Ames et al. (1). Mutagenesis of strain TM677 to 8-azaguanine resistance was carried out as described by Stark et al. (9, 10). To produce dense mutant rings around disks in the forward mutagenesis assay (spot test), 10^8 to 3×10^8 cells were seeded on each plate. Where metabolic activation was required, a rat liver microsomal system was used as described (1) and as specified in figure legends. Mutagens, applied in 20-μl portions, were adsorbed into 6.35-mm filter paper disks (Schleicher and Schuell), and the disks were then placed at the center of each dish. Zones of mutants appeared on the plates after 2 to 4 days of incubation at 37°C. The radii of the external edges of mutant rings were measured. Regression lines and correlation coefficients were calculated as described (Awerbuch and Sinskey, Mutat. Res., in press).

Mutagens and mycotoxins. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) and ethyl methane sulfonate were from Sigma Chemical Co. *N*-Methyl-*N*-nitrosourea was from Pfaltz & Bauer. *N*-Acetoxy dimethyl nitrosamine was kindly provided by S. R. Tannenbaum (Massachusetts Institute of Technology). 1-Nitroso-pyrrolidine was from Aldrich Chemicals, Inc. Nitrosomorpholine was from Research Laboratories.

The mutagenic mycotoxins mollicellins C and E were produced from *Chaetomium mollicellum* solid-substrate fermentation of rice and subsequently purified as previously described (Büchi et al., submitted for publication). The mycotoxins produced by *Penicillium islandicum* Sopp. (rubroskyrin and simatoxin) were isolated by the method of Ghosh et al. (4, 5, 10). Rugulosin was obtained through the courtesy of S. Shibata, University of Tokyo, Tokyo, Japan.

Preparation of mutagen solutions. NG and *N*-methyl-*N*-nitrosourea were each dissolved in absolute ethanol to give stock solutions of 1 and 10 mg/ml. Fresh aqueous solutions were prepared in 0.15 M phosphate buffer (pH 7.0) before each experiment. Similarly, ethyl methane sulfonate, dimethyl nitrosamine, nitrosomorpholine, and 1-nitroso-pyrrolidine were freshly dissolved in the same buffer before use or applied directly on the filter paper. All mycotoxins, including petroleum-ether-insoluble (PEI) fraction of *Chaetomium* fermentation, were freshly dissolved in dimethyl sulfoxide at 1 to 10 mg/ml and were kept at 0°C until used.

RESULTS AND DISCUSSION

Figure 1 shows typical mutant rings formed around disks containing mutagen solution. Because the mutagen NG was applied at decreasing concentration, mutant rings decreasing in di-

ameter appeared. At the lowest NG concentration (Fig. 1C), no growth inhibition was observed. This phenomenon of mutagenicity in the absence of growth inhibitory effects was also observed with simatoxin and with PEI fraction extracted from the mollicellin-producing fungus *C. mollicellum* (not shown). Figure 2 shows the functional relationship between the initial mutagen concentration on the disk and the outer radius of the mutant ring. All the mutagens shown in Fig. 2 (NG, *N*-methyl-*N*-nitrosourea, ethyl methane sulfonate, and *N*-acetoxy dimethyl nitrosamine) do not require metabolic activation. Similar dose-response curves were obtained with 1-nitroso-pyrrolidine and nitrosomorpholine in the presence of rat liver metabolizing systems (Fig. 3). The system yields similar results not only with the various nitrosamines described above, but also with structurally and functionally unrelated mutagens such as the alkylating agent ethyl methane sulfonate (Fig. 2c), with *P. islandicum*-produced mycotoxins that belong to the anthroquinone group (Fig. 4a) and are suspected to be hepatocarcinogens [(+)rugulosin, rubroskyrin, and simatoxin], and with mutagenic *Chaetomium*-produced mycotoxins that belong to the depsidone group (Fig. 4b).

The relationship ln c_0 α r_{mut} is independent of the indicator strain used and the type of mutation scored; the system exhibits the same relationship between the parameters (as expressed in this equation) for the histidine-reversion spot test with strain TA1535, which requires histidine

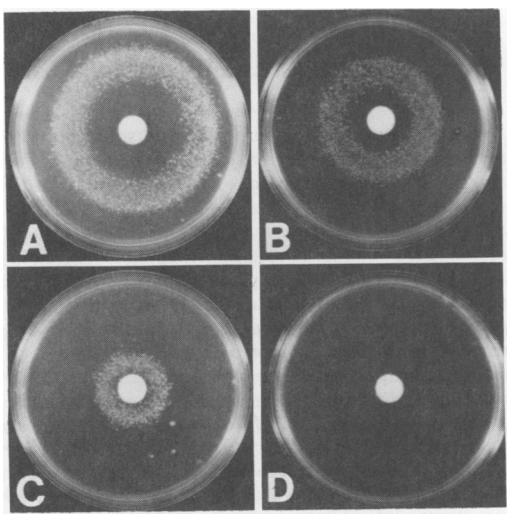

FIG. 1. *Photograph showing the reversion of S. typhimurium TA1535 when 0.025 ml of a solution of NG was placed on a 6.3-mm filter paper in the center of a 60-mm petri plate. The concentration of NG was: (A) 1,000 μg/ml; (B) 100 μg/ml; (C) 10 μg/ml; (D) none.*

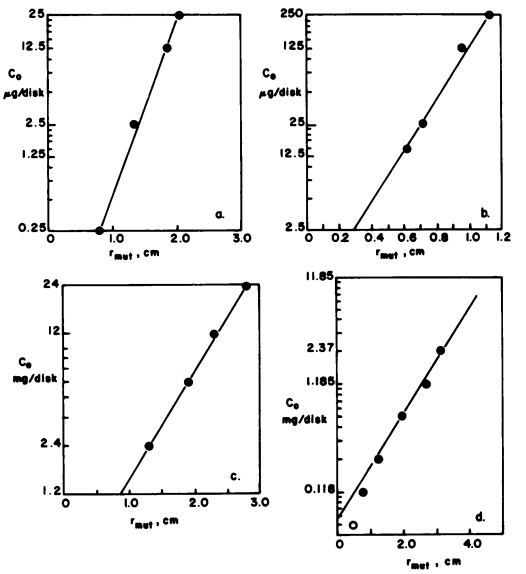

FIG. 2. *The natural logarithm of the initial dose (c_0) of mutagen, placed on a 6.3-mm filter paper at the center of a petri dish containing a lawn of S. typhimurium TA1535, versus the size of the radius of the mutagenic zone, r_{mut} (ln c_0 versus r_{mut}). The radius was measured after 2.5 days of incubation at 37°C. (a) NG: the regression line is $\hat{y} = -7.66 + 3.48\,\hat{x}$, and the correlation coefficient is 1; (b) N-methyl-N-nitrosourea: the regression line is $\hat{y} = -4.27 + 5.97\,\hat{x}$, and the correlation coefficient is 0.993; (c) ethyl methane sulfonate: the regression line is $\hat{y} = -1.38 + 1.59\,\hat{x}$, and the correlation coefficient is 0.999; (d) N-acetoxy dimethyl nitrosamine: the regression line is $\hat{y} = -2.91 + 1.5\,\hat{x}$, and the correlation coefficient is 0.904.*

and does not contain the ampicillin-resistant plasmid pKM101, and for forward mutations to 8-azaguanine resistance in strain TM677, a histidine-prototrophic strain that does contain the plasmid.

We followed the production of a mutagenic substance throughout the fermentation of rice by *Chaetomium*. PEI fractions were prepared from fermentation samples on days 5, 12, and 21 of the fermentation, the solid extracts were dissolved, and various PEI concentrations were tested for mutagenicity as described. Figure 5 shows that the relation ln c_0 α r_{mut} exists at all times during the fermentation. Using a standard curve of mollicellin E (Fig. 4b) to estimate the actual amount of mutagenic compound in crude mixtures (PEI), we derived that the fungal culture produced mutagenic substance equivalent to 6, 7.6, and 16 μg of mollicellin E per ml on days 5, 12, and 21 of the fermentation, respectively. *Chaetomium* produces simultaneously, under the fermentation conditions employed, eight related depsidones (mollicellins A to H).

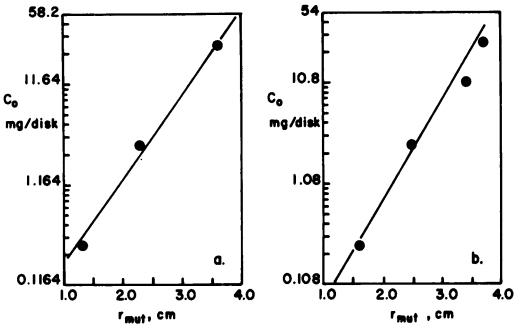

FIG. 3. *The natural logarithm of the initial dose (c_0) of promutagen placed on a 6.3-mm filter paper at the center of a petri dish containing a lawn of S. typhimurium TA1535, incubated with 0.5 ml of an activating system, versus the size of the radius of the mutagenic zone, r_{mut} (ln c_0 versus r_{mut}). The radius was measured after 3 days of incubation at 37°C. (a) Nitrosomorpholine: the regression line is $\hat{y} = -3.83 + 1.99\,\hat{x}$, and the correlation coefficient is 0.995; (b) 1-nitroso-pyrrolidine: the regression line is $\hat{y} = -4.58 + 2.08\,\hat{x}$, and the correlation coefficient is 0.993. The activating system contained, per ml: 8 μmol of $MgCl_2$, 33 μmol of KCl, 5 μmol of glucose 6-phosphate, 4 μmol of nicotinamide adenine dinucleotide phosphate, 100 μmol of sodium phosphate (pH 7.4), and 0.1 ml of rat liver homogenate, fraction S-9.*

Only two of them are mutagenic (9). PEI fractions from solid-state fermentations contain all eight compounds. We chose mollicellin E, the more potent mutagen, as the quantitative standard for expressing the mutagenic potential of crude fractions.

Another useful application of this quantitative spot test is to follow up the decrease in mutagenic potency of solutions of unstable mutagens. A 30-day-old solution of mollicellin E yielded smaller mutant rings (Fig. 4b); however, this partially active solution yielded a dose-response curve similar to that obtained with fresh solution. The two slopes are identical, which indicates that the mutagenic substance has the same decay time and diffusion coefficients in both of them (Awerbuch and Sinskey, in press).

We found theoretically (1a) and experimentally (this work) that linear dose-response curves can be obtained for mutagenic zones of some carcinogens and mycotoxins. This relationship could be used to assess potency tests for mutagens for which other standard methods might not be available, either because the mutagens cannot be purified from the medium or because they are produced in such small quantities that their isolation is practically impossible.

We have shown that the modification of the Ames spot test employed here may be a rapid, easy method for quantitation of mutagens in solution; diameters of mutant rings and initial mutant concentration on paper disks obey a mathematical relationship that allows construction of standard dose-response curves. Those curves may be used to assay mutagen solutions of unknown concentration. The generality and usefulness of this method are indicated by the facts that such a dose-response relationship (i) is valid for many mutagens that are unrelated structurally or by their mode of action, (ii) is not distorted by the incorporation of in vitro microsomal activation systems, and (iii) is independent of the indicator strain used or the type of mutation (forward or reverse) scored. Aside from proper dosimetry of mutagens, we show here that this system is useful in following the development of mutagens in crude preparation and in estimating their degradation (inactivation) dur-

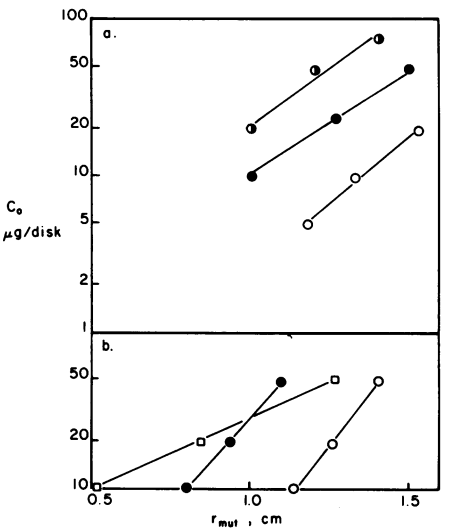

FIG. 4. *Quantitative mutagenesis spot assay of various mycotoxins. 8-Azaguanine resistance forward mutation test using S. typhimurium TM677. Reaction mixtures contained 50 μmol of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.4), 16.5 μmol of KCl, 4.0 μmol of $MgCl_2$, 28 μmol of glucose, and up to 2×10^8 Salmonella cells in a total volume of 600 μl. Thirty milliliters of soft (0.6%) agar was then added, and the mixture was overlaid onto appropriate plates. Paper disks containing the appropriate amount of mycotoxin solution were then placed at the plate centers, and the plates were incubated at 37°C. Mutant zone diameters were measured after 48 to 72 h of incubation. (a) Mutagenicity of mycotoxins produced by P. islandicum Sopp. and by Penicillium rugulosum: (○) rubroskyrin; (◑) simatoxin; (●) (+)rugulosin. (b) Mutagenicity of mycotoxins produced by C. mollicellum: (○), mollicellin E, fresh dimethyl sulfoxide solution; (●) mollicellin E, 30-day-old dimethyl sulfoxide solution, stored at 4°C in the dark; (□) mollicellin C, fresh dimethyl sulfoxide solution.*

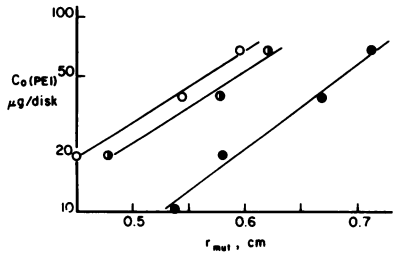

FIG. 5. *Titration of mutagenic activity developing during rice fermentation by C. mollicellum. 8-Azaguanine resistance forward mutation test using S. typhimurium TM677. PEI fractions were prepared from solid-state fermentation of rice with C. mollicellum, as described in detail by B. Kobbe (Master's thesis, Massachusetts Institute of Technology, Cambridge, 1972). PEI fractions were prepared at days 5* (○), *12* (◑), *and 21* (●) *of fermentation. PEI fractions were dissolved in dimethyl sulfoxide shortly before use, and the appropriate amounts were applied to paper disks and placed on seeded plates, as described in the text. Mutant zones were measured after 48 to 72 h of incubation.*

ing storage.

A technique such as the one proposed in this paper is important because of the abundance of carcinogenic substances in our environment (2, 7, 8) that can be screened by this method.

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