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Assay of 855 Test Chemicals in Ten Tester Strains Using a New Modification of the Ames Test for Bacterial Mutagens

Robert E. McMahon,[1] John C. Cline,[2] and Christina Z. Thompson

Lilly Research Laboratories, Indianapolis, Indiana 46206

ABSTRACT

Determination of mutagenic activity in bacterial systems has become accepted as an initial step in the evaluation of the carcinogenic potential of new chemicals. In this paper, a bacterial mutagen screening technique is described in which chemicals can be tested in 10 tester strains over a 10,000-fold concentration gradient both with and without metabolic activation. Using this assay, 855 chemicals were tested, and 182 were found to be mutagenic in one or more of the tester strains. Included were 299 chemicals used in chemical manufacturing or laboratory synthesis. Of these, 20% gave a positive response in one or more strains. The high rate of positives undoubtedly reflects the high chemical reactivity of compounds in this group. In contrast, when 361 organic chemicals which were synthesized for evaluation as potential pharmaceutical or agricultural products were tested, only 8% were identified as mutagenic.

The *Salmonella typhimurium* tester strains TA98 and TA1538 proved to be very reliable and efficient in detecting and identifying frame-shift mutagens. TA100 was the most sensitive tester strain, detecting 142 of the 182 mutagens encountered in the study. However, since TA100 detected both base substitution mutagens and frame-shift mutagens, this tester strain was not suitable for the specific identification of base substitution mutagens. Base substitution mutagens were more reliably detected by *Escherichia coli* tester strains WP2 and WP2 *uvrA*$^-$ than they were by *S. typhimurium* strains G46 and TA1535. The data obtained when mutagens are tested by the concentration gradient procedures can include (*a*) the activity spectrum in tester strains, (*b*) identification as either frame-shift or base substitution mutagens, (*c*) the minimal concentration at which auxotroph growth is inhibited, and (*d*) mutagenic potency in terms of minimal concentration at which mutagenicity is observed. The data obtained have been found to be of immediate use. For example, with manufacturing intermediates the data have been combined with other toxicity data and used as a basis for setting safety standards for handling such compounds in the workplace. In addition, positive bacterial mutagenicity data on selected members of new series of organic compounds can serve to alert the chemist early to the possibility that the compounds may possess undesirable toxic properties, particularly carcinogenicity. Also, this type of data should be of great value both in the planning and in the interpretation of other *in vitro* tests designed to evaluate the potential carcinogenicity in mammals of chemicals found to be positive in bacterial tests.

[1] To whom requests for reprints should be addressed.
[2] Deceased.
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INTRODUCTION

Bacterial test systems for the assay of chemical substances for mutagenic potential are currently broadly used in preclinical toxicology as predictive tests of potential carcinogenicity. Because of their simplicity and minimal time requirements, such tests are nearly irresistible. In particular, the methodology developed by Prof. Ames and associates has enjoyed great popularity. In the procedure of Ames *et al.* (2), the ability of a test compound to revert certain histidine auxotrophs of *S. typhimurium* to the wild type is determined. The sensitivity of the test has been increased by the incorporation of liver enzymes in the test (1) and by the inclusion of new, more sensitive tester strains containing R-factor plasmids (13). Tryptophan auxotrophs of *E. coli* have also been used quite successfully (4, 8). The development of a microbial fluctuation test based on these latter auxotrophs has been particularly promising (7-9). A very large number of compounds have been tested in the various bacterial mutagen tests now in use. For example, McCann *et al.* (12) tabulated results with 300 compounds and concluded that the correlation between *in vivo* carcinogenicity and bacterial mutagenicity is excellent. More recent papers by McCann and Ames (11) and by Sugimura *et al.* (15) support this conclusion.

The successful use of bacterial mutagen testing in a large industrial laboratory requires a test procedure that can efficiently handle many samples and at the same time be sensitive and reliable. To cope with this problem, a method has been developed which allows assessment of the mutagenic potential of chemicals in 8 histidine auxotrophs of *S. typhimurium* and 2 tryptophan auxotrophs of *E. coli* over a 10,000-fold concentration range both with and without metabolic activation (5). This procedure has now been used to test 855 compounds derived from a variety of sources. A summary of the results and some tentative conclusions are presented.

MATERIALS AND METHODS

Tester Strains. The following auxotrophs are derived from *S. typhimurium* LT-2: G46 (histidine$^-$, missense), TA1535 (G46 with *gal-bio-uvrB* deletion and LPS deletion), TA100 (G46 with *gal-bio-uvrB* deletion and LPS deletion with the addition of R-factor pKM101), C3076 [histidine$^-$, (+) frame-shift], TA1537 (C3076 with *gal-bio-uvrB* deletion and LPS deletion), D3052 [histidine$^-$, (+) frame-shift], TA1538 (D3052 with *gal-bio-uvrB* deletion and LPS deletion), and TA98 (D3052 with *gal-bio-uvrB* deletion and LPS deletion with the addition of R-factor pKM101).

The following tryptophan auxotrophs are derived from *E. coli*: WP2 (*E. coli* tryptophan$^-$) and WP2 *uvrA*$^-$ (*E. coli*

tryptophan$^-$ with *uvrA* deletion).

In special cases, 4 additional strains are used for supplemental testing. These strains are TA92 (G46 with the addition of R-factor pKM101), TA94 (D3052 with the addition of R-factor pKM101), CM881 (WP2 with the addition of R-factor pKM101), and CM891 (WP2 with *uvrA*$^-$ deletion and with the addition of R-factor pKM101).

Maintenance Medium. Cultures are maintained in Trypticase soy broth (Baltimore Biological Laboratory) with 0.5% yeast extract (Difco Laboratories) added.

Test Medium. The minimal agar medium was prepared as follows. Each liter of medium contains 14 g of K_2HPO_4, 6 g of KH_2PO_4, 2 g of $(NH_4)_2SO_4$, 1 g of sodium citrate, 0.4 g of $MgSO_4 \cdot 7H_2O$, 2.0 g of glucose, and 10 g of Difco agar (purified). Trace amounts of histidine, tryptophan, and biotin are added immediately before use.

Bacterial Mutagen Screen on Gradient Plates. Agar plates containing a concentration gradient of test compound are prepared as follows. Ten ml of minimal agar medium (not containing test compound) are poured into a square Petri dish (9 x 9 cm) which is tilted at a slight angle. The agar is then allowed to solidify into a wedge-shaped layer by standing at room temperature. Meanwhile, a 1000-μg/ml mixture of test compound in agar is prepared by adding 10 ml of minimal agar to 0.1 ml of a 100-mg/ml solution of test compound in dimethyl sulfoxide. When appropriate, water or dimethoxyethane is used instead of dimethyl sulfoxide. The cooled agar plates are then placed on a level surface, and an overlay of the 10 ml of agar containing the test compound is poured onto the plate to form a reversed wedge of agar on top of the first wedge. A concentration gradient of compound is produced by allowing the compound in the upper wedge to diffuse into the lower layer for 2 hr at room temperature. The concentration range in this plate is approximately 100 to 1000 μg/ml. Three additional plates with concentration ranges of 10 to 100 μg/ml, 1 to 10 μg/ml, and 0.1 to 1 μg/ml are prepared. A streaking device consisting of 10 sterile 50-μl pipets is next dipped into suspensions of the 10 test strains and allowed to fill by capillary action (see Fig. 1). The pipets are then touched to the upper edge of the gradient and drawn across the plate. A wet trail of inoculum is observed. The plates are then incubated for 48 hr at 37°. The minimal medium used in these studies is supplemented with tryptophan, histidine, and biotin in amounts sufficient to allow growth of only about 6 to 10 generations of auxotroph, which would allow expression of mutagenic events, should they occur. If no mutagenic events occur, a very pale streak of bacterial growth is seen along the inoculation streak. When nonlethal mutagenic events occur, discrete colonies appear in this pale background lawn. The frequency of colony appearance increases along the increasing gradient to a concentration at which maximal mutation occurs. Conversely, frequency decreases along the decreasing gradient to a concentration below which only background lawn is observable. These upper and lower concentrations are reported as the concentration range in which the compound is mutagenic under test conditions (see Table 1). A second effect of a test compound, cytotoxicity, is also observed with many compounds. The concentration range over which the test compound is too toxic to permit growth of the auxotroph can be observed by the absence of growth along the application streak (*i.e.*, no background lawn is observable).

Bacterial Mutagen Screen on Gradient Plates with Metabolic Activation of Test Compound. Enzymatic activation of test compounds is achieved by use of a mixture of rat liver enzymes prepared as follows. All steps are done at 4° or lower, and sterile glassware and solutions are used. Livers are removed from 200-g adult male Fischer rats which had received a single i.p. dose of 500 mg of Aroclor 1254 per kg 5 days previously. The livers are placed in sterile 100-ml graduates containing approximately 50 ml of sterile 1.15 M KCl. The volumes of the livers are determined by displacement volume. Approximately 1 ml of liver is mixed with 3 ml of 1.15 M KCl and minced. The minced mixture is then homogenized in a Potter-Elvehjem apparatus with a Teflon pestle. The homogenate is centrifuged for 10 min at 9000 × *g*, and the supernatant is decanted and saved. One ml of the 9000 × *g* supernatant contains about 250 mg of wet liver. This freshly prepared homogenate fraction is distributed in 2-ml portions (equivalent to 500 mg liver, wet weight) to plastic nitrogen storage tubes (Cooke Pro-vial, 2.0 ml), quickly frozen, and stored at −90°. As required, sufficient enzyme is thawed and kept on ice for use. Unused enzyme is discarded at the end of each day.

A solution is next prepared which contains 0.2 ml 9000 × *g* rat liver supernatant, 4 μmol $NADP^+$, 5 μmol glucose 6-phosphate, 5 μmol $MgCl_2$, 32 μmol KCl, and 50 μmol sodium phosphate buffer (pH 7.4) per ml. Sufficient mix is made to allow 0.5 ml for each plate to be used in the test and is held at 4°. The final agar overlay mixture is made as follows: 10 ml of 1% agar containing traces of histidine and tryptophan, and an excess of biotin at 45° are mixed in a test tube with 2 ml of the enzyme mixture and immediately dispensed to 4 plates by pipets. Under these conditions, the microsomes are at a temperature about 40° for less than 1 min. This process is repeated for each multiple of 4 plates in sequence until all plates are overlayed. The plates are streaked in the usual manner and incubated for 48 hr at 37°. The plates are read in the same manner as described above for the nonactivated phase of the test.

Controls. In addition to plates containing test compounds, each test group in both the nonactivated and the activated phases contains 2 sets of positive control plates. One set contains streptozotocin, which is mutagenic without microsomal activation, and the other set contains 2-acetylaminofluorene, which is mutagenic only when activated by liver microsomes. Plates containing no compound are also prepared to serve as negative controls.

Procedure for Testing Volatile Liquids for Mutagenicity. A separate procedure is used to screen certain volatile liquids. The procedure was described by Cline, Thompson, and McMahon at the Ninth Annual Meeting of the Environmental Mutagen Society in March 1978. However, since the abstracts of this meeting are not readily available, a brief description is given.

Square Petri dishes are prepared with a minimal agar base and a sterile coverslip is placed on the agar surface at the center of the top edge. The 10 tester strains are then streaked downward from the coverslip with the applicator developed for the gradient plates (Fig. 1). On the coverslip

of each of 5 plates is then placed a 0.25-inch fiber pad, and compound is added at 5 volumes (0, 2.5, 5, 10, and 20 μl). After a 48-hr incubation period, plates are scored by noting increased numbers of mutant colonies over background in much the same way that the gradient plates are read. For the detection of mutagens requiring enzymatic activation, plates are used in which the minimal agar base is overlayed with 3 ml of agar containing the 9000 × *g* rat liver supernatant fraction. Epichlorohydrin served as a positive control. It gives a positive response in TA1535, TA100, WP2, and WP2 *uvrA*$^-$ without activation and in addition is also active in G46, TA1538, and TA98 with activation.

RESULTS

Methodology

Gradient plate techniques have frequently been used in industrial microbiology for assessing the effects of test chemicals on microorganisms. The method seemed ideally suited for the development of the modification of the Ames test described in this paper. The basic difference between the 2 methods is the scoring of results. In the Ames methods, numbers of mutant colonies are counted and compared with spontaneous mutation rates. In the gradient technique, the concentration range over which chemically induced mutant colonies are present is recorded. Fig. 2 shows 2 plates at the stage at which the operator reads the result. The control plate (*left*) is typical. The number of spontaneous mutant colonies appearing on the auxotrophic lawn streak varies from 0 to 2 in all cases except for TA100 (third streak), which shows a much higher spontaneous rate. The plate to the *right* contains a concentration gradient of streptozotocin of 0.01 μg/ml at the *bottom* to 0.1 μg/ml at the *top*. On this plate, mutation is observable in G46, TA1535, TA100, TA1537, WP2, and WP2 *urvA*$^-$. As examples of the type of data recorded, G46 would be considered to be active over a concentration range of 0.02 to 0.1 μg/ml, while the range for TA1537 would be 0.04 to 0.1 μg/ml. In practice, when the plates are read the operator has a series of 4 plates covering a 10,000-fold concentration range on which to base his judgment. If the test chemical is toxic to the auxotroph, minimal inhibitory concentrations can be observed as clear zones and are also recorded.

So that the concentration gradient technique can work satisfactorily, it is essential that the gradient form properly. Although past experience indicated that most organic compounds diffuse readily through agar, it seemed prudent to confirm this point with a selection of key compounds. This was accomplished as follows. Duplicate sets of plates were prepared: one set in which the test compound was in the bottom wedge and had to diffuse upward and one in which it was in the upper wedge and had to diffuse downward. The plates were then processed and read in the usual manner. The results are shown in Table 1. The correlation between the 2 sets of plates is remarkably consistent in almost all cases with respect to both mutagenic concentration and minimal inhibitory concentration values for those compounds which inhibit auxotrophic growth. One compound which performed poorly was benzo(*a*)pyrene, which diffuses very slowly in agar, making it difficult to work with in most modifications of the Ames test. Since the gradient test is a safety evaluation test, the test compounds are always placed in the upper wedge. Thus, if a compound does not diffuse downward properly it will still be present in the top layer and will be exposed to the tester strain in any event, an important feature for a safety test.

The basic limitation of the gradient method compared to the standard Ames procedure should be clearly understood. The data obtained in the Ames test is in terms of mutant colonies/plate and can be converted to mutation frequencies for comparison with the background rate. Such data can be evaluated by classical statistics and handled easily by standard data-processing routines. The data obtained by the gradient method is in terms of concentration ranges and is much less readily handled by such techniques. In addition, the reading of the plates involves more operator judgment and therefore requires more experienced personnel. The advantage of the gradient method is its high capacity. For example, one compound can be evaluated in 10 tester strains over a 10,000-fold concentration range both with and without metabolic activation using only 8 agar plates. With the standard Ames test, using 5 concentrations, 100 plates would be required to obtain the same data. By the gradient method, one laboratory worker can test about 18 new compounds/week.

Selection of Tester Strains and the Classification of Chemical Mutagens

A battery of 10 tester strains was selected for the primary screen. Eight of these were histidine auxotrophs kindly supplied by Prof. B. N. Ames. The remaining 2 were tryptophan auxotrophs obtained through the courtesy of Dr. M. H. L. Green and Dr. B. A. Bridges. Five strains, D3052, TA1538, TA98, C3076, and TA1537, are considered to be frame-shift mutants and should detect frame-shift mutagens. The remaining 5 strains, G46, TA1535, TA100, WP2, and WP2 *uvrA*$^-$, are presumed to be reverted to prototrophy by base substitution mutagens. The relationships between the various tester strains used in these studies are shown in Chart 1. As has been noted by others and is apparent from the results reported herein, the response to TA100 is somewhat ambiguous since it responds to mutagens of both types. Table 2 lists some of the compounds encountered in the present study which appear to be base substitution mutagens. As expected, many alkylating agents or mutagens such as the nitrosamines which are metabolized to alkylating agents appear here. Less obvious, however, are compounds such as the sydnones, benzimidazole propionic acid, and acetamidrazone. Four of the compounds, butylene oxide, propylene oxide, diethyl sulfate, and epichlorohydrin, were more reliably detected by assay in the vapor phase than in agar gradients. Hydrazine gave a strong reproducible response when agar gradients of the free base (rather than the salt form) were tested in the presence of liver enzymes.

Table 3 lists a few of the frame-shift mutagens detected. Except for the acridine derivative, these mutagens are most reliably detected by TA98 and TA1538. The response of C3076/TA1537 to 9-aminoacridine is typical of the response

Table 1

Gradient comparison of compounds in upper versus lower wedge

Compound	Location of compound	Tester strain									
		G46	TA1535	TA100	C3076	TA1537	D3052	TA1538	TA98	WP2	WP2 *uvrA*⁻
Compounds not requiring activation											
2-Nitrofluorene											
MCR[a]	U			0.01–6		0.01–0.5	0.01–100	0.01–25	0.01–3	1–80	0.1–100
	L			0.01–7		0.03–0.6	0.04–100	0.01–20	0.01–5	5–100	0.1–100
MIC	U		4	2		0.5			4		
	L		5	4.5		0.7			6		
Phenylsydnone											
MCR	U			1–400		45–500			60–500	20–1000	20–1000
	L			10–600		50–600			20–300	45–1000	40–1000
MIC	U			700		600			900		
	L			800		700			700		
Ethylmethane sulfonate, MCR	U	30–1000	300–1000	10–1000						40–1000	9–1000
	L	6–1000	500–1000	10–1000						50–1000	5–1000
4-Nitrobiphenyl, MCR	U			0.1–6					0.6–100		
	L			0.1–7					0.7–100		
N-Methyl-*N'*-nitro-*N*-nitroso-guanidine											
MCR	U	0.05–6	0.1–9	0.08–6		0.45–4		0.3–3		0.08–40	0.04–40
	L	0.06–20	0.5–30	0.08–20		0.7–6		0.55–5		0.5–50	0.3–50
MIC	U	7	9	7	5	5	5	6	7	40	40
	L	20	35	30	30	10	10	40	40	50	50
Streptozotocin											
MCR	U	0.01–50	0.05–100	0.05–40	0.1–5	0.06–30		0.3–40		0.06–60	0.02–50
	L	0.04–100	0.06–100	0.06–50	0.5–8	0.4–50		0.05–50		0.1–70	0.06–60
MIC	U	90		50	50	50	40		>100		
	L	>100		70	70	60	60		50		
Compounds requiring activation											
Benzo(*a*)pyrene, MCR	U			0.1–40		0.5–1					
	L			0.5–10							
2-Aminoanthracene, MCR	U			0.01–100				1–75	0.1–80		
	L			0.1–60		0.7–45		0.7–70	0.1–100		
4-Aminobiphenyl, MCR	U			0.1–35			4–100	0.2–20	0.2–40		1–60
	L			0.1–45			3–75	0.9–20	0.55–50		0.9–60
2-Amino-6-methoxybenzothiazole, MCR	U			0.01–100		0.1–100	0.03–100	0.03–100	0.01–100		
	L			0.01–100		0.1–100	0.07–100	0.07–100	0.01–100		
2-Aminofluorene											
MCR	U			0.1–30			0.1–100	0.5–20	0.1–30		1–80
	L			0.1–40			0.85–100	0.7–25	0.4–45		1–100
MIC	U		90	30		30			30		
	L		90	45		40			50		
2-Acetylaminofluorene, MCR	U			1–100			1–100	0.7–100	0.1–100		
	L			0.6–50			4–100	0.7–100	0.65–100		

[a] MCR, mutagenic concentration range (μg/ml); U, upper wedge; MIC, minimal inhibitory concentration (μg/ml) (if not reported, MIC was more than the highest level tested); L, lower wedge.

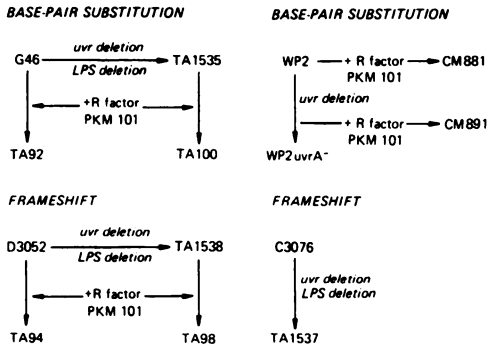

Chart 1. Relationships between tester strains used in this study. G46, D3052, and C3076 are *S. typhimurium* histidine auxotrophs. WP2 is an *E. coli* tryptophan auxotroph.

to other acridines and related compounds. Most of the frame-shift mutagens listed in Table 3 were also positive in TA100, emphasizing the broad sensitivity of this strain.

The division of bacterial mutagens into subgroups is not considered rigorous, nor does it imply knowledge of mechanism of action. It is hoped, however, that it will be useful in helping to interpret results and in planning supplemental testing in other systems. While it is difficult to describe the differences between the 2 classes of compounds in precise terms, some generalizations are possible. For example, the frame-shift mutagens (Table 3) appear to be (*a*) of somewhat higher molecular weight, (*b*) less polar, and (*c*) considerably more planar molecules than the base substitution mutagens (Table 2), indicating that basic differences in mechanism exist. It should be noted that not all bacterial

Table 2

Chemicals detected as base substitution mutagens

	Activity in tester strains which detect									
	Base substitution mutagens					Frame-shift mutagens				
Mutagen	1[a]	2	3	4	5	6	7	8	9	10
Nitrogen mustard	+	+	+	+	+	−	−	−	−	−
Ethylmethanesulfonate	+	+	+	+	+	−	−	−	−	−
6-Bromo-4,4-dimethylcyclohexenone	+	−	+	+	+	−	−	−	−	−
Isobutyraldehyde	+	−	+	+	+	−	−	−	−	−
Benzimidazole-2-propionic acid	−	−	+	+	+	−	−	−	−	−
Homoveratrylsydnone	+	+	+	+	+	−	−	−	−	−
Phenylsydnone	−	−	+	+	+	+	−	−	−	+
Acetamidrozone	+	+	+	+	+	−	−	−	−	−
Nitrosopyrrolidine[b]	+	+	+	+	+	−	−	−	−	−
Diethylnitrosamine[b]	+	−	+	+	+	−	−	−	−	−
Diethanolnitrosamine[b]	+	−	+	+	+	−	−	−	−	−
Dipropylnitrosamine[b]	−	−	+	+	+	−	−	−	−	−
Streptozotocin	+	+	+	+	+	−	−	−	−	−
Propylene oxide	−	+	+	+	+	−	−	−	−	−
Butylene oxide-1,2	−	−	+	−	+	−	−	−	−	−
Hydrazine[b]	−	−	+	+	+	−	−	−	−	−
Epichorohydrin	+	+	+	+	+	−	−	−	−	−
Diethyl sulfate	+	+	+	+	+	−	−	−	+	−

[a] Tester strains: 1, G46; 2, TA1535; 3, TA100; 4, WP2; 5, WP2 *uvrA*⁻; 6, C3076; 7, TA1537; 8, D3052; 9, TA1538; 10, TA98.
[b] Requires microsomal activation.

Table 3

Chemicals detected as frame-shift mutagens

	Activity in tester strains which detect									
	Base substitution mutagens					Frame-shift mutagens				
Mutagen	1[a]	2	3	4	5	6	7	8	9	10
Dimethylaminoazobenzene	−	−	+	−	−	−	−	−	+	+
Acetylaminofluorene	−	−	+	−	−	−	−	+	+	+
2-Aminonaphthalene	−	−	+	−	−	−	−	−	−	−
2-Aminoanthracene	−	−	+	−	−	−	+	−	+	+
2-Aminofluorene	−	−	+	−	−	−	−	+	+	+
9-Aminoacridine	−	−	−	−	−	+	+	−	−	−
4-Aminobiphenyl	−	−	+	−	−	−	−	−	+	+
2-Aminobenzimidazole	−	−	−	−	−	−	−	−	+	+
5-Carbethoxy-2-aminobenzimidazole	−	−	+	−	−	−	−	+	+	+
5-Cyano-2-aminobenzimidazole	−	−	−	−	−	−	−	+	+	+
6-Ethoxy-2-aminobenzthiazole	−	−	+	−	−	−	+	+	+	+

[a] Tester strains: 1, G46; 2, TA1535; 3, TA100; 4, WP2; 5, WP2 *uvrA*⁻; 6, C3076; 7, TA1537; 8, D3052; 9, TA1538; 10, TA98.

mutagens can be so conveniently classified. In particular, as will be seen, aromatic nitro compounds produce positive responses in both types of strains to nearly the same extent.

Structure-Activity Studies

In Table 4, a summary of the results of the testing of 855 compounds is given. The sources of the compounds tested were (*a*) positive and negative control compounds submitted for the purpose of validation of the testing procedure, (*b*) organic intermediates and reagents used in manufacturing and in process development, and (*c*) new organic compounds synthesized specifically for the purpose of testing as potential new products.

The high percentage (21%) of the test compounds which were positive was due in part to the presence of 49 positive aromatic nitro compounds. When only compounds other than aromatic compounds are considered, 133 compounds of 793 tested were positive. Of the 133 positives, 53 (40%) required metabolic activation, an observation which emphasizes the importance of this phase of the assay.

In order to discuss the results of so large and divergent a group of chemicals, they have been subdivided into small groups on the basis of chemical structure. Since the structures of many are quite complex, some compounds are included in more than one group. Aromatic nitro compounds are, however, considered separately and do not occur elsewhere. Many of the compounds tested were of a proprietary nature but were readily classified by chemical type without revealing the full structure. Test results with some of the more interesting chemical types are discussed in the following paragraphs.

Nitro Compounds. A total of 29 nitroimidazoles of varying structures were tested, and all were positive without activation. These compounds, as a group, produced responses in both frame-shift tester strains and base substitution tester strains. An interesting observation is that tester strain D3052, which is repair competent and has an intact cell wall, showed more positive responses than did the corresponding strain, TA1538, which is repair deficient and has an incomplete cell wall. Another observation is that, among the base substitution organisms, the *E. coli* strains detected these compounds quite reliably whereas *S. typhimurium* strains G46 and TA1535 did not. A group of 7 other nitroheterocycles was also tested. All were positive. The most interesting observation in this group was that, of the 3 nitropyrazoles tested, all produced positive responses in

Table 4
Summary of test results of Lilly bacterial mutagen screen

Types of compounds tested	No. tested	Positives found			No. of positive responses in tester strains which detect									
					Base substitution mutagens					Frame-shift mutagens				
		Without activation	Activation required	Total	1[a]	2	3	4	5	6	7	8	9	10
All compounds	855	129	53	182	18	17	142	67	83	6	19	43	63	106
Nitroimidazoles	29	29	0	29	0	0	18	26	27	0	0	18	10	18
Nitropyrazoles, nitrofurans, nitrothiazoles, nitrothiophenes	7	7	0	7	3	0	6	7	7	0	0	0	0	7
Nitroaromatic	26	13	0	13	0	0	12	3	2	0	0	2	7	9
All nitro compounds	62	49	0	49	3	0	36	36	36	0	0	20	17	34
All compounds except nitro compounds	793	80	53	133	15	17	106	31	47	6	19	23	46	72
Aromatic amines and derivatives thereof	79	2	27	29	0	0	23	2	3	0	10	16	25	28
Nitrosamines	10	0	7	7	4	2	6	7	7	0	0	0	0	1
Benzimidazoles, benzthiazoles, benzoxazoles	81	1	27	28	0	0	21	1	1	0	7	13	22	26
Sydnones	4	3	0	3	0	1	3	2	2	1	0	0	0	1
Cinnolines	13	5	0	5	1	0	5	1	1	2	1	1	1	2
Epoxides	4	3	0	3	0	2	3	2	3	0	0	0	0	0
Hydrazides, hydrazines	6	3	1	4	1	1	4	2	4	0	0	0	0	0
Acridines	5	4	1	5	0	0	2	1	2	3	3	1	1	3
Phenazines	8	6	0	6	0	0	3	1	1	0	0	0	6	6
Quinoxalines	6	0	0	0	0	0	0	0	0	0	0	0	0	0
N,*N*-Disubstituted piperazines	20	0	0	0	0	0	0	0	0	0	0	0	0	0
Ergolines	16	0	0	0	0	0	0	0	0	0	0	0	0	0
Monosubstituted pyrimidines	20	0	0	0	0	0	0	0	0	0	0	0	0	0
Pyridines	45	0	0	0	0	0	0	0	0	0	0	0	0	0
Thiazoles	14	0	0	0	0	0	0	0	0	0	0	0	0	0
Thiadiazoles	28	0	0	0	0	0	0	0	0	0	0	0	0	0
Aliphatic nitro compounds	6	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Tester strains: 1, G46; 2, TA1535; 3, TA100; 4, WP2; 5, WP2 *uvrA*$^-$; 6, C3076; 7, TA1537; 8, D3052; 9, TA1538; 10, TA98.

strain G46, which detected none of the other nitroheterocyclic compounds.

Finally, a group of aromatic nitro compounds in which the nitro group was attached to a benzene ring rather than to a heterocyclic ring were tested. Thirteen of 26 compounds tested (50%) were positive. Among the positives were 2-nitrofluorene, 2-nitrophenylenediamine, 3-nitrophenylenediamine, 4-nitrophenylenediamine, 4-nitrobiphenyl, and some nitrophenyl ethers. Negatives included pyrrolnitrin, 5-nitrobenzimidazole, 2- and 3-nitrobiphenyl, and various nitrophenols and nitroanilines. The response of the tester strains to this group of compounds was different than the response to the nitroheterocycles. For example, in this group, tester strain TA100 responded to all of the positives while the *E. coli* tester strains were essentially unresponsive.

In summary, nitro compounds produced positive responses in both frame-shift tester strains and base substitution strains. These compounds are activated directly by the enzymes in the bacteria, and liver enzymes are not required. The results suggest that either the mutagenic metabolite produced by bacterial reduction of the nitro compound is both a frame-shift mutagen and a base substitution mutagen or, alternatively, that 2 active metabolites are produced, one of which is a frame-shift mutagen and one of which is a base substitution mutagen. This matter is being investigated in our laboratory at present.

Nitrosamines. In accord with the results of others, it was found that nitrosamines are detected with difficulty in bacterial test systems. In order to detect this group of compounds reliably, an additional gradient plate containing test compound in the concentration range of 1–10 mg/ml was added. At these concentrations, 7 of 10 nitrosamines were detected. The 3 nitrosamines giving negative responses were diphenylnitrosamine, butylphenylnitrosamine, and propylmethallylnitrosamine. The 7 positive compounds were detected after liver activation as base substitution mutagens. Strains TA100, WP2, and WP2 *uvrA*$^-$ gave the most reliable response. Dimethylnitrosamine and diethylnitrosamine were difficult to detect in the gradient plates because of their volatility but were readily detected by the volatile liquid procedure.

Aromatic Amines. This group, which includes aromatic primary amines, their *N*-alkyl derivatives, and their *N*-acetyl derivatives, contains many of the compounds tested in the validation phase of the test. Positives included 2-acetylaminofluorene, 2-aminofluorene, 2-aminoanthracene, 4-aminobiphenyl, 2-aminonaphthalene, and butter yellow. Negatives included 2-aminobiphenyl, 1-aminonaphthalene, and a large number of variously substituted aniline derivatives. It is well known that mutagens in this class require metabolic activation, presumably by the liver microsomal *N*-hydroxylases. Two compounds, 4-hydroxylaminobiphenyl and 2-hydroxylaminofluorene, were, as expected, active without metabolic activation. Although aromatic amines are well known to be frame-shift mutagens, a positive response in TA100 was noted in 23 of the 29 positives, a further indication of the ambiguous nature of this tester strain.

Benzimidazoles, Benzthiazoles, and Benzoxazoles. A variety of compounds containing benzoheterocyclic rings were tested, and of this group 28 were positive. One, benzimidazole propionic acid, was detected as a base substitution mutagen without activation (Table 2), while an additional 27 compounds were active with metabolic activation. Many of these were analogs of 2-aminobenzimidazole, a compound previously reported to be a bacterial mutagen (14). As is true with other aromatic amines, the structure-activity relationships in this series are quite rigid and not easily predictable. For example, while 2-aminobenzimidazole and 5-cyano- and 5-carbethoxy-2-aminobenzimidazole were active, the corresponding 5-methoxy and 5-carboxy compounds were not active. In the benzthiazole series, 2-aminobenzthiazole as well as 6-cyano-, 4-methyl-, 4-methoxy-, and 6-hydroxy-2-aminobenzthiazole were not active. However, 6-methoxy- and 6-ethoxy-2-aminobenzthiazole were active. All of these compounds require metabolic activation and are frame-shift mutagens. They represent an interesting group of compounds that warrant further investigation. Most of the other active compounds also contained amino groups, but at positions other than position 2.

Other Heterocyclic Compounds. Of the other heterocyclic derivatives tested, 4 classes (sydnones, cinnolines, acridines, and phenazines) contained mutagens. For example, 3 sydnone derivatives (phenyl, orthochlorophenyl, and homoveratryl) were detected as base substitution mutagens without metabolic activation, while sydnone acetamide was negative. The mechanism by which this interesting group of compounds produces a mutagenic response in bacteria is at present unknown. Equally poorly understood is the mutagenic response seen with certain cinnoline derivatives. The acridines are classic frame-shift mutagens and are one of the few groups of compounds that are detected by the tester strain pair C3076 and TA1537. The phenazines are more typical frame-shift mutagens since they are detected by TA98 and TA1538. Interestingly enough, the related 2-ring system, quinoxaline, produced no positives. Other heterocyclic compounds producing no positive responses were the piperazines, ergolines, pyrimidines, pyridines, thiazoles, and thiadiazoles.

Miscellaneous. Among the derivatives of hydrazine, acetyl- and formylphenylhydrazine were detected as base substitution mutagens without activation, as was acetamidrazone (iminoacetylhydrazide). Hydrazine itself was detected as a base substitution mutagen after metabolic activation. Interestingly, hydrazine-free base was used to form the concentration gradient in the test in which hydrazine was positive. When gradients were prepared using salts of hydrazine, a negative result was obtained. Hydrazine derivatives identified as negative included procarbazine, semicarbazide, and benzimidazolehydrazine.

Four aliphatic epoxides were tested. Three of these, propylene oxide, butylene oxide, and epichlorohydrin are chemically very reactive and were detected in this study as base substitution mutagens (Table 2). They are all relatively volatile liquids and were most reliably detected by the volatile-liquid procedure. The data shown in Table 2 on these 3 compounds were obtained by that procedure. Stilbene oxide, a chemically unreactive epoxide, was found to be negative. As with the hydrazides and with the aliphatic nitro compounds, more epoxides must be tested before reliable generalizations are possible.

Relative Response of Tester Strains

As expected, the 2 plasmid-containing strains, TA100 and TA98, proved to be the most sensitive. Thus, 142 of the 182 positives detected were positive in TA100. This included mutagens of both the base substitution type and the frame-shift type. TA98, which was selective for frame-shift mutagens, was mutated by 105 of the 182 positives. It can be concluded that a rapid screen using only these 2 strains would be quite effective. Actually, only 20 (11%) of the 182 mutagens detected in this study were not active in either TA100 or TA98 (see below). The possibility does exist that plasmid-containing strains, particularly TA100, are unrealistically sensitive. In this study, it was noted that 23 of the 142 mutagens positive in TA100 were positive in no other strain. However, this group of 23 did contain a number of important compounds, including difluorophenyl propynyl-*N*-cycloheptylcarbamate, a very potent carcinogen in rats (10); cyclophosphamide; azathioprin; β-naphthylamine; *o*-chlorophenylsydnone, benzyl chloride, *p*-nitrobenzyl bromide, nitrobenzodioxane, and dinitroanisole. Other mutagens active only in TA100 require further investigation before the significance of the positive result is understood. With TA98, only 3 of the 105 positive responses were in TA98 only. In addition to the single responses in either TA98 or TA100 mentioned above, 19 other mutagens were active only in TA100 plus TA98. Thus, a total of 45 (25%) of the 182 mutagens studied were active only in plasmid-containing strains.

As mentioned earlier, one of the more interesting results was the observation that, while the 2 *Salmonella* strains G46 and TA1535 were relatively ineffective in detecting base substitution mutagens, the *E. coli* strains were very effective in this respect. Among the *Salmonella* strains, however, TA100 responded well to these compounds and to frame-shift mutagens also. The usual Ames assay uses 5 strains: TA1535, TA100, TA1537, TA1538, and TA98. If these strains only had been used in the present study, only 16 (9%) of the 182 positives would have been undetected. These 16 include 10 nitroimidazoles which were positive in the *E. coli* strains and in one *Salmonella* strain, D3052. Also included are allyl bromide, chlorophenylaminoethanol, ethyl-2-methallylnitrosamine, and dimethylaminoisopropyl chloride, as well as an unknown impurity in commercial amylene.

Supplemental Tester Strains. Preliminary work on supplemental tests using additional tester strains has been done. One important objective was to find a *Salmonella* tester strain more sensitive than either G46 or TA1535 for detecting base substitution mutagens that would otherwise be detected only by TA100. To do this, the tester strain would have to detect base substitution mutagens with an efficiency similar to that seen with WP2 and WP2 *uvrA*$^-$ and at the same time not be sensitive to frame-shift mutagens that are active in TA100. Preliminary studies to date indicate that TA92 may be such a tester strain, although considerably more work is needed. Work on the plasmid-containing *E. coli* strains CM881 and CM891 has also been done and indicates that these strains may be particularly useful for the detection of nitrosamines. Results presented in this paper indicate that the combination of TA98 and TA1538 is quite adequate for the detection of frame-shift mutagens. However, TA94 appears to offer the possibility of additional confirmation, if needed.

Comparison with Results in the Ames Test

McCann *et al.* (12) have summarized results with 300 compounds assayed by the Ames test. In the current studies, 51 compounds which appear on the Ames list of 300 compounds were also tested. Our results were in excellent agreement with the Ames results in 49 of 51 instances. Two compounds, 1-aminonaphthalene and 2-aminobiphenyl, however, were found to be negative in the present test although they were reported to be weak mutagens in TA100 in the Ames procedure. This result points up a weakness with the test, *i.e.*, the difficulty of reliably detecting small increases in mutation frequency in TA100, which already has a substantially greater spontaneous mutation frequency than do the other tester strains. The same difficulty is frequently experienced with TA100 when the Ames procedure is used.

In additional studies to assess the reliability of the gradient assay, a number of simple derivatives of biphenyl were tested. The results of this study are shown in Table 5. The only unexpected result was the discovery that ethynylbiphenyl has bacterial mutagen properties. This result has been confirmed by further testing of both the compound and of selected analogs. As mentioned above, 2-aminobiphenyl (reported to be weakly active in the Ames test) was inactive in the gradient test. The result in the gradient test may, however, be realistic since no data are yet available showing 2-aminobiphenyl to be carcinogenic in animals.

Synthetic Intermediates and Laboratory Reagents

A total of 299 compounds of the type commonly used in chemical laboratories and in chemical manufacturing were tested. Of these, 60 (20%) gave a positive response in one or more of the tester strains. If it is accepted that the underlying molecular event leading to the observation of bacterial mutagenicity in the test is the chemical interaction of test compound and bacterial DNA, then it is not surprising that this group of highly reactive chemicals should show a high rate of positives. Results with some commonly used laboratory reagents and solvents are listed in Table 6.

New Organic Chemicals Made as Potential New Products

In contrast to the chemical intermediates, which are frequently selected for the very reason that they are highly reactive, the final products of the syntheses are usually more stable, unreactive compounds which can be tested in

Table 5

Results with selected derivatives of biphenyl

Positive in one or more tester strains		
4-Nitro, 4-amino, 4-nitroso, 4-hydroxylamino, 4-ethynyl		
Negative in all 10 tester strains		
4-Bromo	2-Nitro	2-Hydroxy
4-Chloro	3-Nitro	3-Hydroxy
4-Methyl	2-Amino	4-Hydroxy
4-Ethyl	4-Vinyl	3,4-Dihydroxy
4-Isopropyl	4-Carboxymethyl	3,4-Dihydroxy

Table 6
Results with some common reagents and synthetic intermediates

Positives	
p-Nitrobenzyl bromide	Diethyl sulfate
p-Nitrobenzyl acetate	Dimethyl sulfate
Isobutyraldehyde	Hydrazine hydrate
Benzyl chloride	Acrylonitrile
Butylene oxide-1,2	Dibromoethane
Propylene oxide	Dichloroethane
Epichlorohydrin	Sodium azide
Dinitrofluorobenzene	Allyl bromide
Negatives	
Benzsuberone	*p*-Bromoanisole
Methyl vinyl ketone	Acetic anhydride
Anisic acid	Allyl amine
Acetic acid	Benzoyl chloride
α-Pipecoline	Phosphorus trichloride
Homoveratryl amine	Cyclopentanol
Nicotine aldehyde	Octanone-2
Propiophenone	2,6-Dimethoxyphenol
Acetamide	*p*-Methoxyacetophenone
p-Bromopropiophenone	Furfural
Tetrahydrofuran	Benzyl amine
Methyl acrylate	Methane sulfonic acid
	Tetralone-2

biological systems as potential new products. When a group of 361 compounds which were made as potential new pharmaceutical or agricultural products were tested, only 29 (8%) were active. Even this group of 29 presented few unexpected results, since most of them were of the aromatic amine and phenazine frame-shift mutagen type.

Although the group of compounds tested, 361, was a small cross-section of the type of compounds that would be encountered in the testing of new organic compounds, the results do indicate that the number of positives that will be encountered in this type of testing may be quite small.

Minimal Mutagenic Concentrations

It is hoped that ultimately bacterial mutagen assays of various types will not only detect chemical mutagens but will, to some extent, predict potency. The test discussed in this paper gives data not only on the nature of chemical mutagens with respect to the strains in which they are mutagenic but also the concentration range over which they are active. Not including compounds active in the volatile-liquid procedure, 52% of the positive responses were seen in the range of 0.1 to 1 μg/ml, 15% were seen at 1 to 10 μg/ml, 22% were seen at 10 to 100 μg/ml, and only 11% were seen at 100 to 1000 μg/ml. The high percentage of compounds found to be active on the lowest concentration plate is, in part, due to the large number of aromatic nitro compounds tested; they usually respond at very low concentrations. Compounds active only at the highest concentrations include the nitrosamines and certain very insoluble carcinogens such as acetylenic carbamates (10) and cyclophosphamide.

Impurities

A recent paper from the Ames laboratory (6) discusses the very important question of the role of mutagenic impurities in chemicals being tested for mutagenicity in highly sensitive tests such as bacterial mutagen tests. The authors report, for example, that the bacterial mutagenicity of 1-aminonaphthalene and 2-aminobiphenyl can be accounted for, in part, by the presence of the highly mutagenic isomers 2-aminonaphthalene and 4-aminobiphenyl. In the present work, several examples were encountered. For example, an *N,N'*-diaryl urea derivative was tested and found to be a weak frame-shift mutagen with activation. Later, it was observed that one of the starting materials for making this compound, a substituted 2-amino-benzthiazole, was mutagenic with activation at concentrations as low as 10 ng/ml. As a consequence, various lots of the urea were both analyzed for content of starting material and also assayed by the gradient test for mutagenicity. The results (as shown in Table 7) are consistent with the conclusion that the mutagenicity originally observed was due to the presence of unreacted starting materials. The assays shown in Table 7 were performed at different times and not always by the same laboratory worker. The excellent correlation between impurity level and the mutagenic concentration observed is a good indication of the reliability and reproducibility of the concentration gradient technique.

In our experience, it has been worthwhile to investigate unusual positive results with regard to the possibility that impurities may be contributing to the mutagenic response.

Metabolic Activation

In the present study, 53 of the 182 mutagens detected were mutagenic only after metabolic activation by the 9000 × *g* supernatant of liver from rats predosed with Aroclor 1254. Most of these mutagens fall in one of 3 well-recognized groups, the aromatic hydrocarbons, the nitrosamines, and the aromatic amines and their derivatives. The relative importance of each of these 3 groups of compounds is dependent upon the purpose for which the screening work is being done. For example, the polycyclic hydrocarbons and the nitrosamines are probably of greatest interest to environmentalists and public health laboratories. In the industrial setting, however, these compounds are less often encountered than are the frame-shift mutagens of the aromatic amine type. The nature of the enzyme preparation needed to activate each of these 3 groups of compounds is

Table 7
The effect of impurity level upon the mutagenicity of a diaryl urea derivative

Lot designation	Impurity level (ppm)	Concentration range of mutagenicity in TA98 (μg/ml)
A	51,300	0.3–1000
B	11,300	4–1000
C	6,300	30–1000
D	1,300	40–1000
E	237	350–1000
F	128	400–1000
G	120	500–1000
H	10	NM[a]
I	9	NM
J	7	NM
K	3	NM
L	1	NM

[a] NM, not mutagenic over range tested (0.1 to 1000 μg/ml).

probably different. Thus, the choice of an activation system should be made on the basis of the purposes of the assay. It should, of course, be noted that, in addition to the 3 types of mutagens mentioned above, which require metabolic activation, there are a limited number of less frequently encountered chemical types which a bacterial mutagen assay must detect. Included are certain olefins, cyclophosphamide, certain halogen compounds, and the aflatoxins. The activation systems used in the gradient plate assay (as will be discussed in later papers) were characterized before use for their ability to hydroxylate biphenyl. This reaction was chosen since it involves the formation of 2 arene oxide intermediates, one via cytochrome P-448 (leading to 2-hydroxylation) and one via cytochrome P-450 (leading to 4-hydroxylation) and one hydroxylation (position 3) that is thought to proceed in part by direct insertion (3).

DISCUSSION

The assessment of the carcinogenic potential of the very large number of synthetic organic chemicals now in use in commerce and of the new ones to be introduced in the future remains one of the significant challenges in cancer research. Because of the large numbers of chemicals to be tested, short-term *in vitro* tests have gained popularity, particularly the Ames bacterial mutagenicity test. Such tests, of course, cannot be expected to be completely predictive of either carcinogenicity or noncarcinogenicity *in vivo* in mammals. Nevertheless, they can play a very important role in setting priorities for further testing, in signaling the need for safe handling of chemicals in the workplace until further testing has been completed, and as an aid in selecting new chemicals for further development as potential products. In the present paper is described a modification of the Ames technique which enables a laboratory to screen many compounds rapidly in 10 selected tester strains. This procedure has been used to assess 855 compounds drawn from a variety of sources. Some of the conclusions that are possible from the results are recorded above.

The 10 tester strains chosen for the primary screen were divided among organisms thought to revert by base substitution mechanisms and those which revert by frame-shift mechanisms. Through the use of these 10 tester strains, many of the mutagens encountered in this study could be conveniently classified as either base substitution mutagens or frame-shift mutagens. It is hoped that this type of data will be of use in both the planning and the interpretation of results in other *in vitro* and *in vivo* test systems. Some mutagens, particularly the aromatic nitro compounds, appear to possess both characteristics. In the case of the aromatic nitro compounds, which are activated by bacterial enzymes, this implies that the mutagenic metabolite functions by both mechanisms or, alternatively, that there are 2 mutagenic metabolites, one a frame-shift mutagen and one a base substitution mutagen. This possibility is under investigation in this laboratory. Strain TA100 detected both base substitution mutagens and frame-shift mutagens. This strain was also the most sensitive of the 10 tester strains used. A combination of TA100 and TA98 would, as a matter of fact, have detected approximately 90% of the mutagens detected in this study, suggesting that a very rapid screen using only these 2 plasmid-containing tester strains could be useful in some situations. The inclusion of the 2 *E. coli* strains proved to be very useful in the detection of base substitution mutagens since the 2 *Salmonella* tester strains G46 and TA1535 were not efficient in detecting such mutagens.

Because the majority of the compounds tested have not as yet been tested in other *in vitro* systems or in *in vivo* systems, it is not possible to correlate the bacterial mutagenicity of these compounds directly with carcinogenicity. However, the data obtained should form an excellent data base from which to work in the direction of validating further *in vitro* tests and ultimately correlating results with *in vivo* information. Although it has not been possible to make a direct correlation with *in vivo* carcinogenicity for all of the compounds tested, it has been possible to classify almost all of the chemical mutagens encountered in the study into relatively well-defined chemical types related to classes of compounds known to possess carcinogenic properties. Very few of the chemical mutagens detected in this study had chemical structures uniquely different from known carcinogens. Further study in other test systems will be required to assess the significance of results with the few unique compounds encountered. The results of the study do suggest, however, that as testing continues on more and more compounds it will be found that most of the new mutagenic compounds detected will be related to known carcinogens and mutagens and that new unique chemical structures possessing these properties will be found rarely. Another observation from these studies is that the testing of groups of organic chemical intermediates used in chemical manufacture can be expected to produce more positives than the testing of organic chemicals being developed as commercial products, such as pharmaceuticals, agricultural chemicals, and others.

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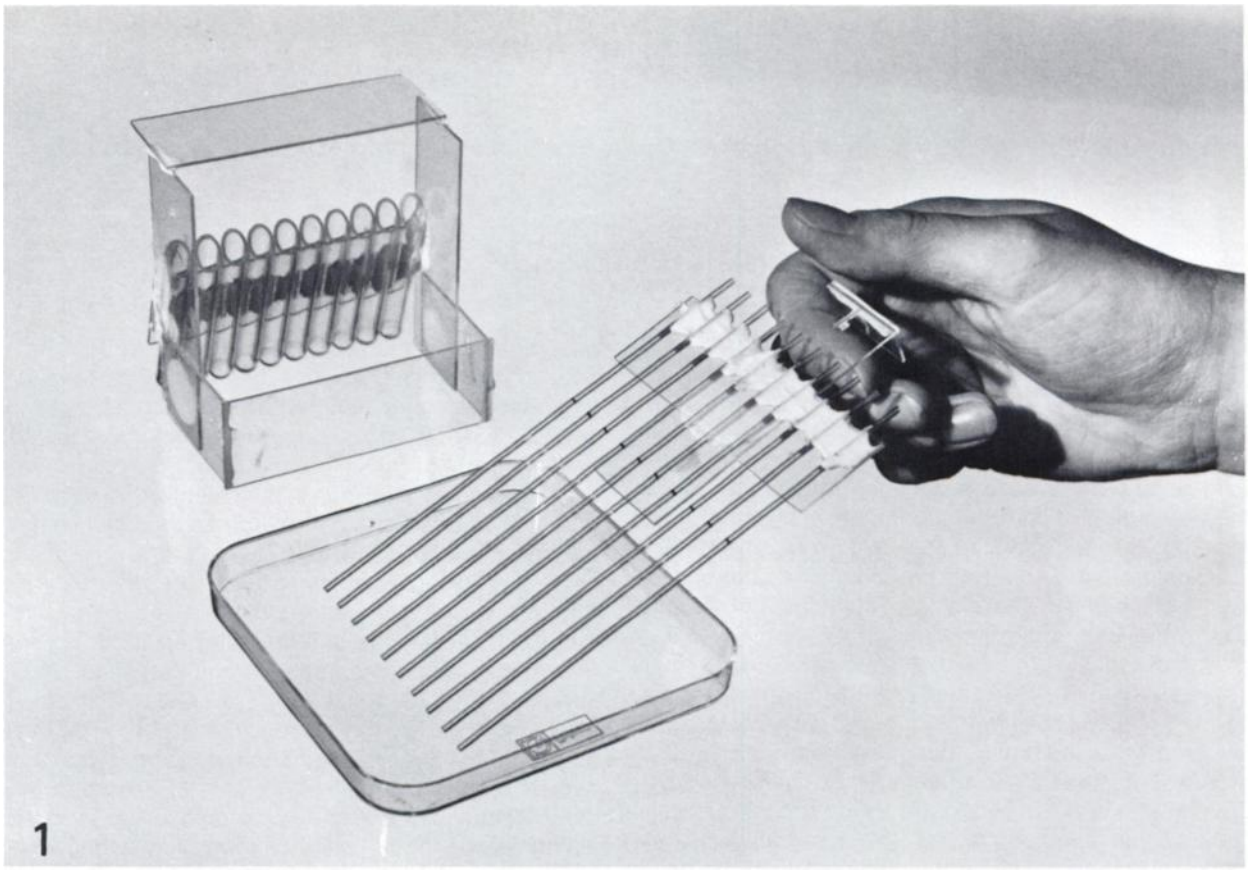
REFERENCES

1. Ames, B. N., Durston, W. E., Yamasaki, E., and Lee, F. D. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc. Natl. Acad. Sci. U. S. A., *70:* 2281–2285, 1973.
2. Ames, B. N., Lee, F. D., and Durston, W. E. An improved bacterial test system for detection and classification of mutagens and carcinogens. Proc. Natl. Acad. Sci. U. S. A., *70:* 782–786, 1973.
3. Billings, R. E., and McMahon, R. E. Microsomal biphenyl hydroxylation: the formation of 3-hydroxybiphenyl and biphenyl catechol. Mol. Pharmacol., *14:* 145–154, 1978.
4. Bridges, B. A. Simple bacterial systems for detecting mutagenic agents. Lab. Pract., *21:* 413–419, 1972.
5. Cline, J. C., and McMahon, R. E. Detection of chemical mutagens. Use of concentration gradient plates in a high capacity screen. Res. Commun. Chem. Pathol. Pharmacol., *16:* 523–533, 1977.
6. Donahue, E. V., McCann, J., and Ames, B. N. Detection of mutagenic impurities in carcinogens and noncarcinogens by high-pressure liquid chromatography and the *Salmonella*/microsome test. Cancer Res., *38:* 431–438, 1978.
7. Green, M. H. L., Bridges, B. A., Rogers, A. M., Horspool, G., Muriel, W. J., Bridges, J. A., and Fry, J. R. Mutagen screening by a simplified bacterial fluctuation test: use of microsomal preparations and whole liver cells for metabolic activation. Mutat. Res., *48:* 287–294, 1977.

8. Green, M. H. L., and Muriel, W. J. Mutagen testing using TRP$^+$ reversion of *Escherichia coli*. Mutat. Res., *38:* 3–32, 1976.
9. Green, M. H. L., Muriel, W. J., and Bridges, B. A. Use of simplified fluctuation test to detect low levels of mutagens. Mutat. Res., *38:* 33–42, 1976.
10. Harris, P. N., Gibson, W. R., and Dillard, R. D. The oncogenicity of two 1,1-diaryl-2-propynyl *N*-cycloalkylcarbamates. Cancer Res., *30:* 2952–2954, 1970.
11. McCann, J., and Ames, B. N. The *Salmonella*/microsome mutagenicity test: predictive value for animal carcinogenicity. *In:* H. Hiatt, J. D. Watson, and J. A. Winsten (eds.), Origins of Human Cancer, pp. 1431–1450. New York: Cold Spring Harbor Laboratory, 1977.
12. McCann, J., Choi, E., Yamasaki, E., and Ames, B. N. Detection of carcinogens as mutagens in the *Salmonella*/microsome test. Assay of 300 chemicals. Proc. Natl. Acad. Sci. U. S. A., *72:* 5135–5139, 1975.
13. McCann, J., Spingarn, N. E., Kobori, J., and Ames, B. N. Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids. Proc. Natl. Acad. Sci. U. S. A., *72:* 979–983, 1975.
14. Seiler, J. P. The mutagenicity of benzimidazole and benzimidazole derivatives. I. Forward and reverse mutations in *Salmonella typhimurium* caused by benzimidazole and some of its derivatives. Mutat. Res., *15:* 273–276, 1972.
15. Sugimura, T., Kawachi, T., Matsushima, T., Nagao, M., Sato, S., and Yahagi, T. A critical review of submammalian systems for mutagen detection. *In:* D. Scott, B. A. Bridges, and F. H. Sobels (eds.), Progress in Genetic Toxicology, pp. 125–140. Amsterdam: Elsevier/North-Holland, 1977.

Fig. 1. Applicator used to apply all 10 tester strains on the plates simultaneously.

Fig. 2. Typical gradient plates after incubation. The 10 tester strain streaks are (from *left* to *right*) G46, TA1535, TA100, C3076, TA1537, D3052, TA1538, TA98, WP2, and WP2 *uvrA*$^-$. The plate on the *left* is a control plate, while the other is a streptozotozin plate with a concentration gradient of 0.01 μg/ml (*bottom*) to 0.1 μg/ml (*top*).



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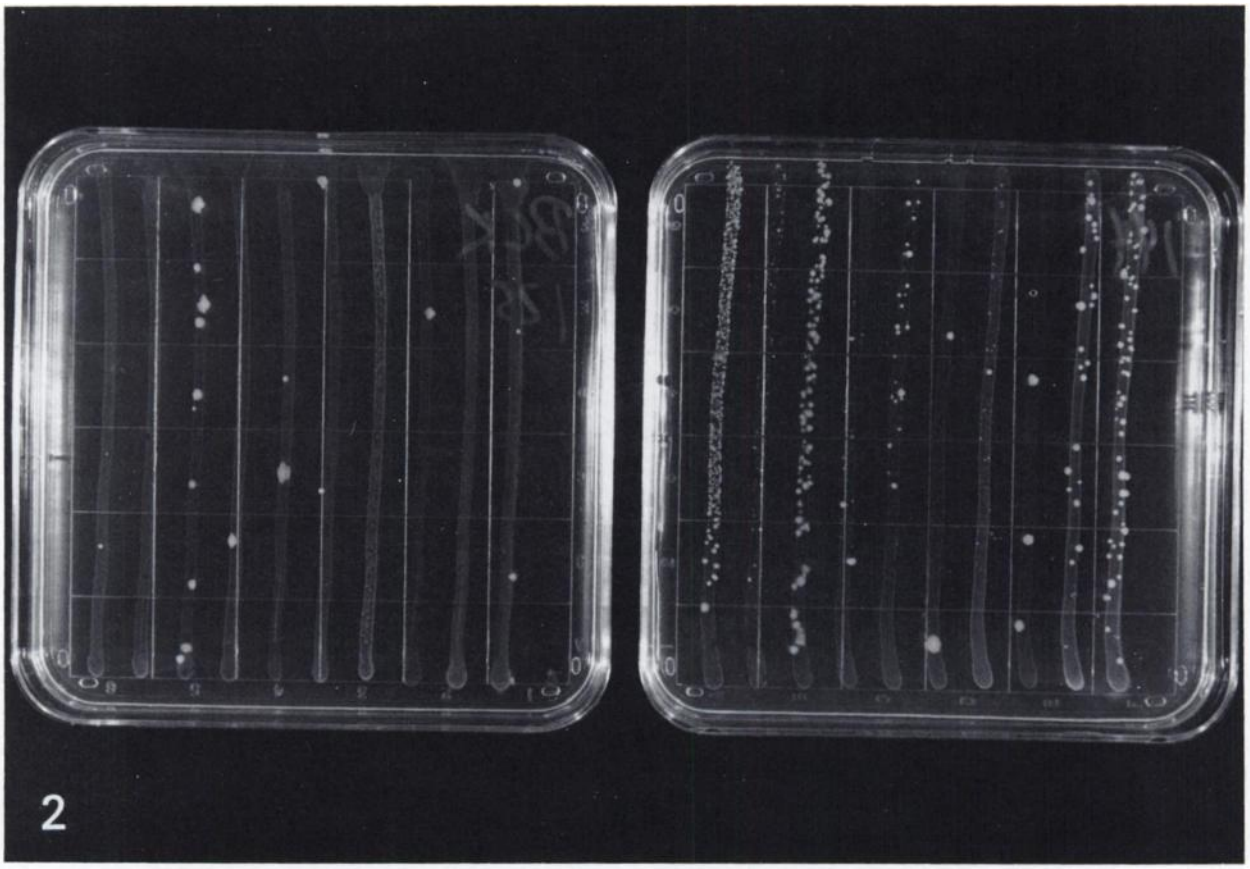

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