

# Revised methods for the Salmonella mutagenicity test

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## Summary

The methods for detecting carcinogens and mutagens with the Salmonella mutagenicity test were described previously (Ames et al., 1975b). The present paper is a revision of the methods. Two new tester strains, a frameshift strain (TA97) and a strain carrying an ochre mutation on a multicopy plasmid (TA102), are added to the standard tester set. TA97 replaces TA1537. TA1535 and TA1538 are removed from the recommended set but can be retained at the option of the investigator. TA98 and TA100 are retained. We discuss other special purpose strains and present some minor changes in procedure, principally in the growth, storage, and preservation of the tester strains. Two substitutions are made in diagnostic mutagens to eliminate MNNG and 9-aminoacridine. Some test modifications are discussed.

## Introduction

The literature on the Salmonella test has grown enormously since the publication of the original methods paper, and test data on more than 5000 chemicals have been published (Environmental Mutagen Information Center Index, 1982). The test has also been used to determine the mutagenicity of complex environmental and

biological mixtures. Many of the mutagenic components of these mixtures have been characterized chemically. A considerable number of mutagens first detected by the Salmonella test have been shown subsequently to be carcinogenic in animal tests. These mutagens include the hair dye amines (NCI Bioassays, 1978a, 1979a, 1979b; Weisburger et al., 1978), 1-nitropyrene from diesel exhaust (Ohgake et al., 1982), the flame retardant, Tris(2,3-dibromopropyl)phosphate (NCI Bioassay, 1978b), and several of the protein pyrolysis products produced by cooking foods (Sugimura, 1982).

The Salmonella test was first validated in a study of 300 chemicals, most of which were known carcinogens (McCann et al., 1975a; McCann and Ames, 1976; McCann and Ames, 1977). It was subsequently validated in studies by the Imperial Chemical Industries (Purchase et al., 1976), the National Cancer Center Research Institute in Tokyo (Sugimura et al., 1976), and the International Agency for Research on Cancer (Bartsch et al., 1980). Nearly 90% of the carcinogens tested were mutagenic in these studies, but there was considerable overlapping of chemicals tested. In a recent analysis, Rinkus and Legator (1979, 1981) concluded that the correlation between carcinogenicity and mutagenicity is lower than the earlier estimates. Ames and McCann (1981), in a discussion of this analysis, currently estimate the correlation to be about 83%. All the validations show that the test fails to detect a few classes of carcinogens such as polychlorinated pesticides (Rinkus and Legator, 1979, 1981; Ames and McCann, 1981). Some of the carcinogens that were reported to be negative can now be detected using the new tester strains or the modifications discussed in this paper. For example, the addition of cofactors to the S9 mix allows the detection of certain azo dyes. It is important to recognize the limitations of the assay as well as the advantages, and, ultimately, a battery of tests is needed so that the strengths of one test can compensate for the inadequacies of another in detecting specific classes of mutagens.

The revised methods provide the essential guidelines for mutagenicity testing and allow sufficient flexibility for the incorporation of test improvements and modifications to fit specific circumstances. In some instances, more strictly defined protocols than presented here may be required, as in the testing of environmental samples by commercial or contract testing laboratories. The protocols for these laboratories may be established by the EPA or other regulatory agencies.

### **The bacterial tester strains**

A set of histidine-requiring strains is used for mutagenicity testing. Each tester strain contains a different type of mutation in the histidine operon (see Table 1). In addition to the histidine mutation, the standard tester strains contain other mutations that greatly increase their ability to detect mutagens. One mutation (*rfa*) causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increases permeability to large molecules such as benzo[*a*]pyrene that do not penetrate the normal cell wall (Ames et al., 1973a). The other mutation (*uvrB*) is a deletion of a gene coding for the DNA excision repair system, resulting

TABLE 1

## GENOTYPES OF THE TA STRAINS USED FOR MUTAGENESIS TESTING

Histidine mutation				LPS	Repair	R-factor
hisD6610	hisD3052	hisG46	hisG428 (pAQ1)			
his01242 = TA88						
TA90	TA1538	TA1535	-	<i>rfa</i>	$\Delta uvrB$	- R
[TA97]	[TA98]	[TA100]	-	<i>rfa</i>	$\Delta uvrB$	+ R
-	TA1978	TA1975	-	<i>rfa</i>	+	R
TA110	TA94	TA92	-	+	+	+ R
-	TA1534	TA1950	-	+	$\Delta uvrB$	- R
-	-	TA2410	-	+	$\Delta uvrB$	+ R
TA89	TA1964	TA1530	-	$\Delta gal$	$\Delta uvrB$	- R
-	TA2641	TA2631	-	$\Delta gal$	$\Delta uvrB$	+ R
-	-	-	[TA102]	<i>rfa</i>	+	+ R

Tester strains in brackets are recommended for general mutagenesis testing. All strains were originally derived from *S. typhimurium* LT2. Wild-type genes are indicated by a +. The deletion ( $\Delta$ ) through *uvrB* also includes the nitrate reductase (*chl*) and biotin (*bio*) genes. The  $\Delta gal$  strains and the *rfa/uvrB* strains have a single deletion through *gal chl bio uvrB*. The *rfa* repair<sup>+</sup> strains have a mutation in *galE*. R = pKM101. The tester strain TA1536, included in the original tester set (Ames et al., 1973a), and all other strains containing the histidine mutation *hisC207* have been discontinued as they are reverted by only a few mutagens and these can be detected well by other tester strains. TA97 replaces TA1537 and TA2637. Genotypes of these discontinued strains and of other derivatives of *hisC3076* can be found in Table 1 of Ames et al. (1975b).

in greatly increased sensitivity in detecting many mutagens (Ames, 1971, 1973a). For technical reasons, the deletion excising the *uvrB* gene extends through the *bio* gene and as a consequence, these bacteria also require biotin for growth. TA102 does not contain the *uvrB* mutation because it was constructed primarily for detecting mutagens that require an intact excision repair system. The standard tester strains, TA97, TA98, TA100, and TA102 contain the R-factor plasmid, pKM101. TA102 also contains the multicopy plasmid, pAQ1, which carries the *hisG428* mutation and a tetracycline resistance gene. These R-factor strains are reverted by a number of mutagens that are detected weakly or not at all with the non R-factor parent strains (McCann et al., 1975b; Levin et al., 1982a). In both *Escherichia coli* and *Salmonella typhimurium*, pKM101 increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system which is normally present in these organisms (McCann et al., 1975b; Walker and Dobson, 1979; Shanabruach and Walker, 1980).

**DNA sequence specificity.** The *hisG46* mutation in TA100 and TA1535 is in the *hisG* gene coding for the first enzyme of histidine biosynthesis (Ames, 1971). This mutation, determined by DNA sequence analysis, substitutes  $\begin{matrix} -GGG- \\ -CCC- \end{matrix}$  (proline) for  $\begin{matrix} -GAG- \\ -CTC- \end{matrix}$  (leucine) in the wild-type organism (Barnes et al., 1982). TA1535 and its R-factor derivative, TA100, detect mutagens that cause base-pair substitutions, primarily at one of these G-C pairs. The *hisD3052* mutation in TA1538 and TA98 is in the *hisD* gene coding for histidinol dehydrogenase. TA1538 and its R-factor

derivative, TA98, detect various frameshift mutagens. Frameshift mutagens can stabilize the shifted pairing that often occurs in repetitive sequences or 'hot spots' of the DNA, resulting in a frameshift mutation which restores the correct reading frame for histidine synthesis. The *hisD3052* mutation has the sequence  $\begin{matrix} \text{-GGCGCGGC-} \\ \text{-CGCGCGCG-} \end{matrix}$ , 8 repetitive -GC- residues near the site of a -1 frameshift mutation in the *hisD* gene (Isono and Yourno, 1974; Barnes and Husson, 1982). This mutation is reverted by mutagens such as 2-nitrofluorene and daunomycin. The new frameshift strain, TA97, replaces the less sensitive TA1537 that was previously included in the standard set of strains. This new strain has an added cytosine resulting in a run of 6 cytosines at the site of the *hisD6610* mutation (Levin et al., 1982a). The mutagenic specificity of TA97 is similar to that of the *hisC3076* mutation in TA1537 but because TA97 also has a second hot spot of alternating -GC- base pairs near the run of cytosines, it is sensitive to some of the mutagens that revert TA1538 and TA98. TA102 contains the ochre mutation,  $\begin{matrix} \text{-TAA-} \\ \text{-ATT-} \end{matrix}$ , in the *hisG* gene. This strain detects efficiently a variety of mutagens such as formaldehyde, glyoxal, various hydroperoxides, bleomycin, phenylhydrazine, X rays, UV light, streptonigrin, and cross-linking agents such as psoralens and mitomycin C. These mutagens are not detected or are detected poorly by the standard set of tester strains.

**Primary tester strains.** For general mutagenicity testing we recommend primarily TA97, TA98, TA100, and TA102. TA1535 and TA1538 can be used in addition to TA100 at the option of the investigator. TA1535 has a considerably lower spontaneous mutation frequency than TA100 and is thus more convenient for the detection of mutagens that do not preferentially revert TA100 (McCann et al., 1975b). TA1537 is replaced by TA97 but is available on request. Although TA1538 is useful for the detection of particular aromatic frameshift mutagens such as 4-nitro-*o*-phenylenediamine, we decided to drop the strain because it overlaps considerably with TA98. Herbold (1982), reporting preliminary data from an international survey on sensitivity of the tester strains, concludes that TA1538 can be dropped from the tester set on the grounds that it does not furnish significant information beyond that obtained with the other strains. The new tester strain, TA102, is discussed separately by Levin et al. (1982b).

**Adjunct strains.** *HisG46* is more sensitive than TA1535 or TA100 for hard methylating agents such as dimethylnitrosamine or MNNG.

TA94 (*hisD3052/pKM101*) was developed to detect DNA cross-linking agents such as mitomycin C which require the R-factor and an intact excision repair system. Mitomycin C was negative in tests with the original set of tester strains (Ames and Haroun, 1980). TA102 is much more sensitive than TA94 for detecting mitomycin C (Levin et al., 1982b), and further testing may show that TA94 is superfluous.

TA2637 (TA1537/pKM101) is more sensitive than TA1537 for detecting certain frameshift mutagens (Jose, 1979). This strain has also been replaced by TA97.

Strain TA1978 (*hisD3052/rfa*) can be used in combination with TA1538 in a *Repair test* which indicates whether an agent is killing bacteria by damage to DNA that can be repaired by the *uvrB* excision repair system (Ames, 1972; Ames et al., 1973a).

Other recently constructed adjunct strains are discussed in Levin et al. (1982a, 1982b).

Nitroreductase-deficient derivatives of TA100, TA98, and TA1538 were isolated by Rosenkranz and Speck (1975, 1976). These are useful for studying the metabolism and mutagenicity of nitro carcinogens such as nitrofurazone and furylfuramide which are activated directly to mutagens by bacterial nitroreductases. Rosenkranz and Speck (1975, 1976) used nitroreductase-deficient strains to demonstrate the mutagenic activation of some nitro-containing therapeutic agents and showed that mammalian liver nitroreductases can also activate this class of carcinogens. Nitrated pyrenes are extremely potent mutagens (Rosenkranz et al., 1980a) that have been detected in diesel emissions (Xu et al., 1982; Schuetzle et al., 1980, 1981). Recently, McCoy et al. (1981) have developed a new derivative of TA98, designated TA98/1,8DNP<sub>6</sub>, which is deficient in the nitroreductase specific for nitropyrenes. The original nitroreductase-deficient strains should not be used for testing nitropyrenes (Rosenkranz et al., 1981).

### Procedure for growing cultures

Tester strain cultures are grown in Oxoid nutrient broth No. 2 to a density of  $1-2 \times 10^9$  cells per ml. The amount of culture required for a mutagenicity assay depends on the size of the experiment and is based on 0.1 ml of culture per plate. For small experiments we grow 5 ml of culture in  $18 \times 150$  mm culture tubes with loose fitting caps. If larger volumes are required, flasks or culture bottles can be used. The bottles should have loose fitting caps. Flasks can be covered with sterile paper cups or metal foil. The capacity of the vessel should be 3-5 times the volume of the culture. Cultures are inoculated from master plates or from frozen permanents and are incubated in a 37° gyrorotary incubator. To insure adequate aeration, cultures are shaken at approximately 210 rpm. When flasks are used, the rate of rotation should be decreased to about 120 rpm to avoid foaming. The procedures for making master plates and frozen permanents are described in the section, *Storage of the tester strains*. Nutrient broth is discussed in the section, *Spontaneous reversion*. We previously specified that the growth period should not exceed 16 h (Ames et al., 1975b). This interval was largely a matter of convenience and we have subsequently found that viability decreases in most nutrient broth cultures grown longer than 12 h. To shorten the growth period, the incubator can be connected to a timer set to turn on automatically about 10 h before the cultures are needed. The freshly inoculated broth cultures are placed in the incubator at room temperature at the end of the day and the cultures are removed in the morning as soon as they have reached the specified density, determined by turbidity measurements at 650 nm. The culture tubes can be placed in wood block carriers or wrapped with metal foil to protect the bacteria from light although there is some question whether this is necessary in normal laboratory conditions. De Serres and Shelby (1979) recommend placing the cultures in an ice bath when they are removed from the incubator. This is appropriate for storing the cultures until they are needed for the assay because there is

some loss of viability in nutrient broth cultures of *rfa* strains that remain at room temperature for several hours. However, we maintain the cultures at room temperature during the mutagenicity assay to avoid thermal shock to the bacteria when they are placed in 45° top agar.

### Confirming genotypes of tester strains

The tester strain genotypes should be confirmed (a) immediately after receiving the cultures, (b) when a new set of frozen permanents or lyophilized cultures is prepared, (c) when the number of spontaneous revertants per plate falls out of the normal range or (d) when there is a loss of sensitivity to standard mutagens. In many laboratories, the tests for genotypes are included in each mutagenicity assay.

Fresh broth cultures are used for these tests. All reagents, glassware, petri plates, inoculating sticks, and cotton swabs are sterile. See the *Recipe* section for the preparation of reagents and growth media.

**Histidine requirement.** The His<sup>-</sup> character of the tester strains is confirmed by demonstrating the histidine requirement for growth on selective agar plates. Biotin is also required by all of the standard tester strains (except TA102) because of the *uvrB* deletion which extends through the *bio* gene. These nutrients can be added to minimal glucose agar before the test plates are poured (see histidine/biotin plates in the *Recipe* section) or they can be applied to the surface of minimal glucose agar plates and incorporated into the agar with a glass spreader. For each plate, 0.1 ml of 0.1 M L-histidine and 0.1 ml of 0.5 mM biotin are required. The first solution applied to the plate should be spread thoroughly until it is completely taken up by the agar before the second solution is applied. Control plates contain biotin but no histidine. It is not necessary to prepare control plates without biotin for testing TA102. The plates can be wrapped in plastic bags and stored at 4° for about 2 months.

**Procedure:** Dip a cotton swab, wire loop, or wooden stick into the culture and make a single sweep across the biotin control plate and then across the histidine/biotin plate. 5 or 6 strains can be tested on each plate. The strains are identified by labelling each streak with a marking pen on the bottom of the petri plate. If a cotton swab is used, excess culture should be squeezed out on the inside of the culture tube to avoid transferring nutrients from the broth to the minimal plates. The plates are incubated overnight at 37° and examined for growth on the histidine/biotin plates. There should be no growth on the control plates.

It is not necessary to test for the biotin requirement because the mutation that deletes the *bio* gene cannot be reverted. Moreover, testing for biotin dependence has caused some confusion because growth is frequently seen on the control plates, particularly when swabs are used to apply the cultures. This is because the requirement for biotin is extremely small and can be satisfied by traces present in the nutrient broth that saturates the swab. To do this test properly, the nutrient broth should be removed from the culture by centrifugation (3000 rpm for 15–20 min) and decantation followed by resuspension of the pellet in phosphate-buffered saline.

*rfa* mutation. Strains having the deep rough (*rfa*) character should be tested for crystal violet sensitivity (Ames et al., 1973a). For this test, nutrient agar plates are seeded with cultures of the strains to be tested and a sterile filter paper disc containing crystal violet is placed on the surface of each seeded plate.

*Procedure:* For each tester strain, add 0.1 ml of a fresh overnight culture to a tube containing 2 ml of molten top agar held at 45°. It is not necessary to add histidine and biotin. Vortex for 3 sec at low speed and pour on a nutrient agar plate. Tilt and rotate the plate to distribute the top agar evenly, place it on a level surface, and allow several minutes for the agar to become firm. Pipet 10  $\mu$ l of a 1 mg/ml solution of crystal violet to the center of sterile filter paper discs (1/4 inch) and transfer one disc to each of the seeded plates using sterile forceps. Press the disc lightly with the forceps to embed it slightly in the overlay, taking care not to move it laterally. Invert the plate and incubate at 37°C. After 12 h incubation, a clear zone of inhibition (approximately 14 mm) appears around the disc indicating the presence of the *rfa* mutation which permits large molecules such as crystal violet to enter and kill the bacteria. Wild-type strains or strains containing the *gal* deletion (Table 1) are not inhibited because the crystal violet cannot penetrate the cell.

*uvrB* mutation. The *uvrB* mutation can be confirmed by demonstrating UV sensitivity in strains that contain this mutation (Ames et al., 1973a).

*Procedure:* With sterile swabs, streak the tester strain cultures across a nutrient agar plate, in parallel stripes. Non-R-factor strains should be streaked on a separate plate. Place a piece of cardboard over the uncovered plate so that half of each bacterial streak is covered. Irradiate the plate with a 15-W germicidal lamp at a distance of 33 cm. Non-R-factor strains (TA1535, TA1537, and TA1538) are irradiated for 6 sec and R-factor strains (TA97, TA98 and TA100) are irradiated for 8 sec. A strain with wild-type excision repair enzymes (e.g., TA102) should be tested on the same plate as a control for the UV dose. Incubate the irradiated plates at 37°C for 12–24 h. Strains with the *uvrB* deletion will grow only on the un-irradiated side of the plate. Regular monitoring of the UV lamp with an actinometer is advised because the energy output decreases over a period of time.

*R-factor.* The *R-factor* strains (TA97, TA98, TA100, and TA102) should be tested routinely for the presence of the ampicillin resistance factor because the plasmid is somewhat unstable and can be lost from the bacteria (McCann et al., 1975b). It should be emphasized that ampicillin resistance has nothing to do with the increased sensitivity of the R-factor strains to reversion by mutagens. For purposes of the test it is simply a convenient marker that makes it possible to test for the presence of the plasmid. Specific regions of the pKM101 DNA that are essential for enhancement of UV and chemical mutagenesis, replication, and ampicillin resistance have been identified (Langer et al., 1981).

*Procedure:* To test for ampicillin resistance, streak the cultures across the surface of an ampicillin plate (see the *Recipe* section) using the procedure described above for confirming the histidine requirement. Several strains can be tested on the same plate. A non-R-factor strain should be tested on the same plate as a control for ampicillin activity. After incubation for 12–24 h at 37°, there should be growth along the streaks made with the R-factor strains and no growth along the control

streak. Zeiger et al. (1981) recommend commercial ampicillin discs which are applied to plates seeded with the bacteria as in the test for the *rfa* mutation. The absence of zones of inhibition around the discs indicate resistance to ampicillin. Experiments confirming the increased mutagenesis of TA100 compared to TA1535 with methyl methanesulfonate (or nitrofurantoin) and of TA98 compared to TA1538 with daunomycin, are also recommended (McCann et al., 1975b). Positive controls are routinely included for all the tester strains used in a mutagenicity assay.

*pAQ1 plasmid.* The *pAQ1 strain* (TA102) should be tested for both ampicillin and tetracycline resistance on ampicillin/tetracycline plates. See the *Recipe* section for preparation of plates. An R-factor strain is used as a control for tetracycline.

### Spontaneous reversion

Spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenicity experiments and is expressed as the number of spontaneous revertants per plate. The revertant colonies are clearly visible in a uniform background lawn of auxotrophic bacteria. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. The number of revertants that arise spontaneously during the 48-h incubation is dependent on the final number of auxotrophs on the plate and that number is a function of the histidine concentration. We emphasize that the number of spontaneous revertants per plate is completely independent of the initial number of bacterial cells plated, within the limits, roughly, of  $10^5$  to  $10^8$  cells (Green and Muriel, 1976). The auxotrophs (background bacteria) are not counted but their number is assumed to be constant because the histidine concentration is constant. Nevertheless, there is variability in the number of spontaneous revertants from one experiment to another and from one plate to another, and it is advisable to include at least 3 spontaneous mutation control plates for each strain in a mutagenicity assay. This is essential when the test compounds are weak mutagens. Acceptable ranges of spontaneous reversion may be somewhat different in different laboratories but they should be relatively consistent within a laboratory. The following ranges are based on historical values and are therefore more reliable for TA98 and TA100 than for the new strains (TA97, TA102) which have been in use for only a short time. Revertants/plate (-S9): TA97 (90-180), TA98 (30-50), TA100 (120-200), TA102 (240-320). The numbers may be slightly different on plates with S9.

Somewhat different ranges of spontaneous reversion may be observed over a long period of time but there should not be extreme fluctuations from one experiment to the next. A deviation that is obviously outside the acceptable range is an indication that the genetic characteristics of the strain in question (or the growth medium) should be tested. Abnormally high spontaneous reversion may indicate contamination or the accumulation of back mutations by repeated sub-culturing, in which case the strain may be recovered by reisolation from the frozen master copy. See the following section for the reisolation procedure.

A decrease in the spontaneous reversion frequency of the R-factor strains

accompanied by sensitivity to ampicillin, and a corresponding insensitivity to the appropriate diagnostic mutagens, are indications of R-factor loss. This can be partial or complete. The R-factor titer can be monitored and complete loss of the plasmid prevented by growing an overnight culture (from the frozen master copy) in Oxoid broth to which 25  $\mu\text{g}/\text{ml}$  ampicillin has been added. To determine the proportion of cells that have retained the R-factor, dilute an overnight culture  $10^6$  times with sterile phosphate-buffered saline (0.02 M phosphate, 0.15 M NaCl, pH 7.4) and plate 0.1 ml in triplicate, on nutrient agar plates with and without ampicillin. Incubate overnight at 37° and score the plates. The average number of colonies on the ampicillin plates should be equal to the number on plates without ampicillin, indicating that all the bacterial cells carry the R-factor plasmid. If necessary, the strain can be reisolated from ampicillin plates. See the *Reisolation* section.

Spontaneous reversion is also influenced by the histidine concentration and consequently, fluctuations in the histidine content of the top agar will be reflected in corresponding fluctuations in the number of spontaneous revertants on the plates. See the *Top agar* section. Consistently high spontaneous values not attributable to the histidine concentration can sometimes be traced to mutagens in the environment of the bacteria (see ethylene oxide, below).

*Ethylene oxide* used to sterilize petri plates and other disposable plastic ware is a potent mutagen for TA1535 and TA100. Residues of ethylene oxide in the plates can greatly increase the numbers of revertant colonies of these strains. Petri plates sterilized with ethylene oxide should not be used in mutagenicity assays. See the *Agar plates* section.

*Nutrient broth* from Difco was previously specified for growing cultures of the tester strains (Ames et al., 1975b). By 1978, however, we were seeing a doubling of the spontaneous reversion frequency of TA1535 and TA100. The problem was traced to the nutrient broth (Maron et al., 1981) which induced mutations in TA1535 and TA100 without S9 activation. Mutagens for TA1538 and TA98, requiring S9 activation, have also been detected in Difco nutrient broth (Vithayathil et al., 1978). We have had conflicting reports from other laboratories about the problem of high spontaneous mutation with Difco broth but we have not investigated it further and we now use Oxoid nutrient broth No. 2 for growing the tester strain cultures. See *Chemical sources*. Unfortunately, any nutrient broth that contains proteins extracted from beef at high temperatures is likely to be mutagenic to some extent.

### Reisolation of tester strains

Tester strains are reisolated from the frozen master copies by streaking the bacteria on minimal glucose agar plates enriched with histidine and biotin. For reisolating R-factor strains, the agar should contain ampicillin in a concentration of 25  $\mu\text{g}/\text{ml}$ . For reisolating TA102, tetracycline (12  $\mu\text{g}/\text{ml}$ ) is also added. See the *Recipe* section for preparation of these plates.

*Procedure:* Dip a sterile wooden stick into a freshly thawed permanent culture (or

scrape bacteria from the surface of the frozen culture) and make a single streak across the surface of the agar plate. Cross-streak the culture using a sterile platinum wire. Incubate the plate for 48 h at 37°C. With a sterile wire loop or wooden stick, pick a well-isolated colony for overnight growth in Oxoid nutrient broth No. 2. New frozen permanents can be prepared from these liquid cultures according to the procedures described below.

### Storage of the tester strains

*Frozen permanent* copies of the tester strains are stored at  $-80^{\circ}$ . They are prepared from fresh overnight cultures to which DMSO is added as a cryoprotective agent.

*Procedure:* Grow a fresh Oxoid nutrient broth culture to a density of  $1-2 \times 10^9$  bacteria per ml. For each 1.0 ml of culture, add 0.09 ml of spectrophotometric grade DMSO. Combine the culture and DMSO in a sterile tube, flask or bottle, according to the number of permanents to be prepared. Swirl gently until the DMSO is dissolved and distribute the culture aseptically into sterile 1.2 ml cryotubes (Nunc) which have been labeled appropriately. The tubes should be filled nearly full, allowing for expansion due to freezing. This eliminates the air space at the top and helps to minimize oxidative damage. Place the tubes upright in a bed of crushed dry ice until the cultures are frozen solid and then transfer to a  $-80^{\circ}\text{C}$  freezer. We recommend making multiple copies of each strain. One or more master copies should be set apart to be opened only for regenerating the frozen stocks. The other copies are used sequentially for inoculating the overnight cultures used in mutagenicity assays or for making master plates. See the *Master plates* section, below. In our laboratory, each frozen permanent is opened many times to remove bacteria by scraping the surface with a sterile wooden applicator stick but this procedure may be inadvisable in certain circumstances; for example, if many people have access to the permanents or if the freezer is badly placed with respect to airborne contaminants. When permanents are opened more than once, care must be taken not to contaminate the cultures and they should in no circumstances be allowed to thaw. Many problems with the tester strains arise from leaving the frozen permanents out of the freezer too long. To prevent thawing, the permanents may be placed in a bed of crushed dry ice but they should be returned to the freezer as soon as possible. If the freezer is located some distance from the laboratory, with no facilities for inoculating cultures under sterile conditions, it is advisable to use one of the alternative procedures given in the next paragraph. After a permanent has been used for several months or opened 5-10 times, it is probably well to discard it and open another. We use master plates for the frequently used standard tester strains. Overnight cultures of infrequently used strains are inoculated directly from the frozen permanents.

If freezer space is available, large numbers of permanents can be prepared once or twice a year. In this case, one frozen permanent of each tester strain is sacrificed for each mutagenicity assay and master plates are eliminated. The frozen cultures are thawed at room temperature and a measured amount is used for inoculating the

overnight cultures used in the assay. A 20- $\mu$ l inoculum is recommended for each 5 ml of broth. An alternative approach requiring fewer frozen permanents is to sacrifice one permanent each time a master plate is prepared, every two months. Whenever a permanent is thawed for making master plates or for inoculating overnight cultures, the remaining thawed culture must be discarded.

*Viability of frozen permanents.* We have used the same frozen permanents for 3 years and have had no problems with viability or loss of genetic markers. These permanents have been opened approximately once each month during the 3-year period. On the other hand, several of our original frozen permanents, prepared in 1974, grew very poorly after storage at  $-80^{\circ}$  for 5 years. Survival may have been jeopardized by opening these permanents too many times during this 5-year period.

*Liquid nitrogen* has been used for storing frozen permanents in laboratories that do not have  $-80^{\circ}$  freezers. We have no experience with liquid nitrogen storage.

*Master plates.* In addition to frozen permanents, it is convenient to have cultures of the tester strains on master plates which can be stored at  $4^{\circ}\text{C}$ . These are minimal glucose agar plates enriched with histidine and biotin. Ampicillin is added to plates used for the R-factor strains, and for TA102, tetracycline is also added (see the *Recipe* section). Master plates are used routinely in our laboratory as the source of bacteria for inoculating the overnight cultures of frequently used strains. The use of these plates for routine work avoids the problems that arise when the frozen permanents are opened frequently. Each person in the laboratory has his own set of master plates.

*Procedure:* Thaw a frozen permanent (or scrape bacteria from the surface). Apply one drop of thawed culture to the surface of a histidine/biotin plate and streak out for single colony isolation using a sterile platinum wire. Incubate 48 h at  $37^{\circ}\text{C}$ . With a sterile wire loop, remove a well-isolated colony and suspend it in 0.3 ml or less of phosphate-buffered saline contained in a small culture tube. Dip a sterile cotton swab into the bacterial suspension, squeeze out the excess on the inside of the tube and make 4 or 5 parallel streaks across the surface of the appropriate agar plate. Incubate overnight at  $37^{\circ}$ . Reisolating the strains before making master plates maintains the spontaneous reversion frequency characteristic of the strains.

Master plates are stored at  $4^{\circ}$  and are discarded after 2 months or sooner if the number of spontaneous revertants per plate falls out of the range specified for a strain. For reasons we do not understand, the spontaneous reversion frequency of TA97 and TA100 increases appreciably when master plates are too old. TA102 master plates should be discarded after 2 weeks because the spontaneous reversion frequency increases as a consequence of the tetracycline added to the agar to retain the pAQ1 plasmid. This increase may be caused by selection for a higher copy number of the pAQ1 plasmid and does not appear to be an indication of tetracycline mutagenicity (Levin et al., 1982a). Because of this problem, master plates are not practical for storage of TA102 and we recommend using frozen permanents as the source of bacteria for inoculating liquid cultures. If one wants to use TA102 master plates despite their lack of longevity, the spontaneous reversion frequency should be monitored carefully. It is essential to keep master plates cold at all times. In our laboratory, they are stored in the cold room ( $4^{\circ}$ ) and are not removed for inoculat-

ing cultures. If a cold room is not available, master plates can be stored in a conventional refrigerator but they should be removed only to inoculate cultures and should be returned immediately thereafter. New master plates are never made from the old ones because of the risk of losing the *rfa* mutation or the plasmids through subculturing.

*Liquid cultures.* We previously specified that liquid cultures of the tester strains can be stored in the refrigerator for 1 week (Ames et al., 1975b) but subsequent experiments with Oxoid cultures stored at 4° indicate that the viability drops significantly after 1 day with strains carrying the *rfa* mutation. We now recommend using only fresh liquid cultures for mutagenicity assays.

*Lyophilized permanents.* If a -80° freezer is not available, the strains may be stored as lyophilized cultures. These permanents are prepared from skim milk suspensions of the tester strain cultures. The cultures, harvested from LB agar plates, are lyophilized, sealed under vacuum and stored at 4°. All solutions, glassware, cotton swabs, petri plates, etc. are sterile. See the *Recipe* section for preparation of media and reagents used in the procedure described below.

*Procedure:* Dip a cotton swab into a fresh Oxoid broth culture of the tester strain and swab the entire surface of several LB agar plates. The swab should be damp but not completely saturated. Incubate the plates, inverted, for about 16 h at 37°. Survival is better from cultures that have reached the stationary phase than from cultures harvested in the growth phase (LaPage et al., 1970).

*Harvesting the cultures:* Add 1.6 ml of 10% sterile skim milk to the culture on one of the plates. The volume of skim milk is somewhat flexible but should be kept small because survival of the lyophilized culture is proportional to the size of the population in the suspension medium and therefore a relatively dense suspension of bacteria should be used (LaPage et al., 1970). Suspend the culture in the skim milk using a glass spreader. Tip the plate to allow the culture to collect at the lower rim and transfer it to a test tube using a Pasteur pipette. This should be done quickly so that the milk does not soak into the agar. Repeat this procedure for all of the plates, combining the milk suspensions. Mix by vortexing gently. Set aside approximately 0.3 ml of the culture suspension to be tested for viability of the bacteria.

*Lyophilization:* Transfer 0.2 ml of the bacterial suspension to a long neck vacuum ampule, taking care not to deposit any of the culture on the neck of the ampule. This can be done, with a little practice, using a disposable micropipette. Only one aliquot needs to be measured accurately in order to test for viability after lyophilization. The others may be transferred to the ampules with a Pasteur pipette, approximating 0.2 ml by eye (8-10 drops). Freeze the samples quickly in an alcohol-dry ice bath, rotating the ampule to distribute the culture evenly over the sides of the ampule while avoiding the neck. Lyophilize for about 8 h after a vacuum of 5-10 millitorrs has been reached.

*Sealing the ampules:* Seal the ampules under vacuum with a two-way oxygen torch. Check each ampule for maintenance of vacuum using a high frequency spark generator and discard any samples that are not under vacuum. Label the samples and store in the dark at 4°.

One of the lyophilized cultures for each strain should be tested for viability and

integrity of genetic markers. The loss of viability due to lyophilization is usually about 90%. The 0.2-ml sample should retain  $2 \times 10^8$  or more viable bacteria. The genetic markers are tested by growing an Oxoid culture from one of the lyophilized samples and testing as described in the section on tests for genotypes.

*Longevity of lyophilized cultures:* We have stored freeze-dried cultures of TA100 for 2 years with good survival of the bacteria and no loss of the genetic characteristics.

## Mammalian liver S9

*Induction of rat liver enzymes.* For general mutagenesis screening we recommend liver homogenates from rats induced with a polychlorinated biphenyl (PCB) mixture, Aroclor 1254 (Alvares et al., 1973; Czygan et al., 1973; Ecobichon and Comeau, 1974; Litterst and Van Loon, 1974; Schmoltdt et al., 1974). The induction procedure (Kier et al., 1974) is similar to the methods of Czygan et al. (1973). We use Sprague-Dawley male rats weighing approximately 200 g. Aroclor 1254 is diluted in corn oil to a concentration of 200 mg/ml and a single i.p. injection of 500 mg/kg is administered to each rat 5 days before sacrifice. The rats are given drinking water ad libitum and Purina Laboratory Chow until 12 h before sacrifice when the food (but not the water) is removed. On the 5th day of induction the rats are killed by cervical dislocation. The procedures for removing the livers from rats and preparing liver homogenates are described in the following sections.

On occasion we have used other tissues and other species, but in general rat liver is the most convenient source of activating enzymes. For efficient detection of a wide variety of carcinogens requiring metabolic activation, it is essential that S9 be prepared from induced animals. We have used various induction procedures (Ames et al., 1973b; Kier et al., 1974) and find that liver from Aroclor-induced rats is efficient for detecting different classes of carcinogens. To demonstrate the relative efficiencies of different induction procedures, we compared S9 fractions from uninduced, phenobarbital-, 3-methylcholanthrene-, and Aroclor 1254-induced rats for the activation of two polycyclic hydrocarbons, benzo[*a*]pyrene and 3-methylcholanthrene, and an aromatic amine carcinogen, 2-acetylaminofluorene. The results showed that S9 preparations from induced rats are superior to those from uninduced rats for the activation of the three compounds tested (Ames et al., 1975b). Aroclor 1254 induction was selected as the best compromise for the detection of these three carcinogens. Although phenobarbital is a generally effective inducer and can be used for the efficient detection of 2-acetylaminofluorene and many other aromatic amine carcinogens (Ames et al., 1973b), it is very inefficient for detection of certain polycyclic hydrocarbons.

Aroclor is a carcinogen of great stability and the 1 billion pounds of PCBs in the environment have caused concern. The disposal of PCB-containing animal carcasses adds in a minor way to this problem. More importantly, Aroclor may be a greater potential hazard to laboratory workers than other carcinogens: it is difficult to handle because of its viscosity and it is present in small amounts in the S9 (Shahin et

al., 1979). For these reasons it would be beneficial to have an alternative to Aroclor for S9 induction. Matsushima et al. (1976) compared two induction procedures and found that a combined injection of phenobarbital and  $\beta$ -naphthoflavone produced induction similar to Aroclor-1254. They proposed the use of this combination as a substitute for Aroclor-1254. Ong et al. (1980) compared S9 induction following treatment of rats with several inducers and also found the combination of phenobarbital and  $\beta$ -naphthoflavone to be an effective alternative for PCB induction.

*Removal of liver from rats.* To insure a clean S9 preparation, the livers must be removed aseptically using sterile surgical tools.

*Procedure:* Kill the animal by cervical dislocation and place it on its back on an autopsy board. Secure the feet with pins. Swab the fur thoroughly with 70–95% ethanol or tincture of iodine. Cut through the skin using sterile pointed scissors or scalpel. If the skin is lifted from the underlying layers, the incision can be made without cutting into the muscle. Fold back the skin flaps and pin them to the autopsy board to avoid getting fur into the abdominal cavity. Swab the muscle layer with ethanol and then cut through this layer with a fresh pair of sterile scissors or scalpel taking care not to cut into the esophagus or intestines as this will result in contamination of the liver homogenate. Excise the livers.

*Preparation of liver homogenate S9 fraction.* Preparation of the liver S9 fraction is based on the procedure of Garner et al. (1972). All steps of the procedure are carried out at 0–4° using cold, sterile solutions and glassware. The freshly excised livers are placed in preweighed beakers containing approximately 1 ml of chilled 0.15 M KCl per g of wet liver. One rat liver weights approximately 10–15 g. After weighing, the livers are washed several times in fresh, chilled KCl. Successive washings in KCl are essential to insure a sterile preparation and to remove hemoglobin which can inhibit the activity of the cytochrome P450 enzymes. The washed livers are transferred to a beaker containing 3 vol of 0.15 M KCl (3 ml/g wet liver), and are minced with sterile scissors, and homogenized in a Potter-Elvehjem apparatus with a teflon pestle or with a Polytron homogenizer. The homogenate is centrifuged for 10 min at 9000 g (8700 rpm in SS-34 rotor of Sorval RC2-B) and the supernatant (the S9 fraction), is decanted and saved. Sterility of the preparation is determined by plating 0.1 ml on minimal agar containing histidine and biotin. 1 ml of S9 fraction contains microsomes from about 250 mg of wet liver. The protein concentrations are approximately 40 mg/ml as determined by the procedure of Lowry et al. (1951), and are fairly constant from one batch to the next. The freshly prepared S9 fraction is distributed in 1–2 ml portions in small plastic Nunc tubes, frozen quickly in a bed of trushed dry ice, and stored immediately at –80°C. If large experiments are anticipated, 5-ml portions may be frozen. The S9 required for a mutagenicity assay is thawed at room temperature and placed in a container of crushed ice. S9 mix should be made as soon as the S9 has thawed. Sterility of the S9 mix is determined by adding 0.5 ml to top agar and plating on minimal glucose agar. Although it is possible to remove most contaminants by filtration of the S9 mix through a 0.45- $\mu$  filter, this procedure introduces the risk of enzyme loss, particularly if there is foaming. It should not be necessary to filter the S9 mix if the livers are removed under aseptic conditions as described above.

*S9 from other tissues and animals.* Microsomal preparations can be made from various tissues of other species. We have compared liver and lung from rats (Kier et al., 1974) and rat and human autopsy liver (Ames et al., 1973b, 1975a). S9 preparations from mouse and hamster liver have also been made. These preparations were similar to preparations from rat liver except for problems of sterility with lung tissue and homogenization difficulties with lung and human liver. Because of its fibrous nature, lung tissue was homogenized at 0–4°C for 5 min using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) instead of the Potter–Elvehjem apparatus (Kier et al., 1974). The Polytron is useful for any tissue that is fibrous or difficult to homogenize. The S9 fraction for these tissues was prepared from the homogenate in the same manner as the liver fraction. Although liver preparations were usually sterile, lung preparations contained bacterial contaminants. These were removed by passage of the lung S9 mix through a sterile Swinex filter unit (Millipore Corp., Bedford, MA) equipped with a 0.45- $\mu$  millipore filter. If the filter clogs, a larger pore size (0.8  $\mu$ m) can be used which removes almost all of the bacteria and gives a better flow rate. For heavily contaminated preparations, this initial filtration should be followed by a second filtration using a smaller pore size. Nalgene disposable membrane filter units can be used for large amounts of S9 mix. The S9 fraction cannot be filtered before it is diluted into the mix because it

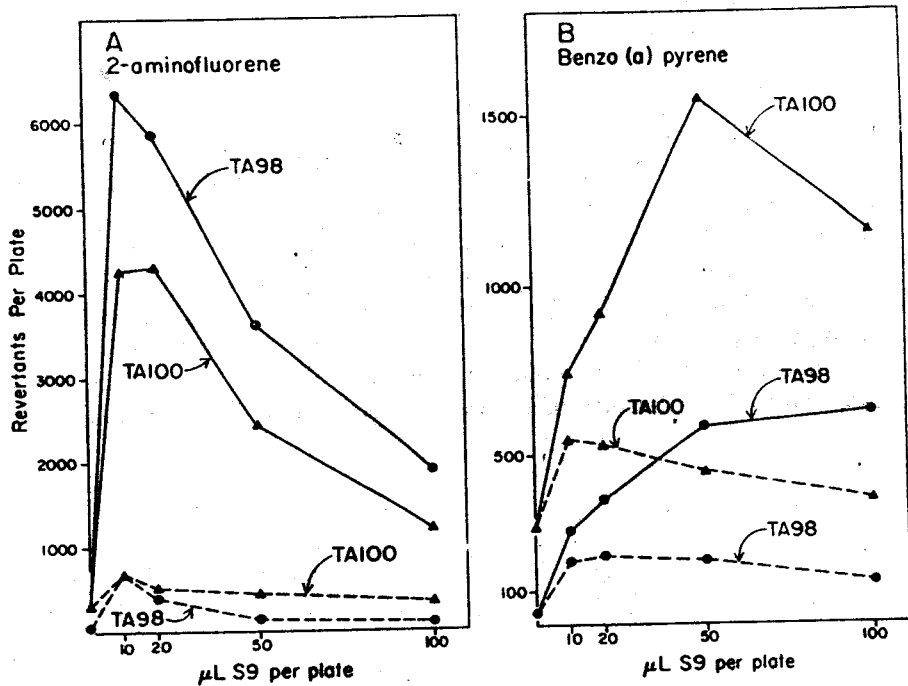


Fig. 1. The effect of increasing concentrations of S9 and a constant dose of mutagen on the mutagenesis of TA98 and TA100. Liver S9 was from Aroclor 1254-induced rats. (A) 2-aminofluorene: solid lines, 20  $\mu$ g; broken lines, 1  $\mu$ g. (B) Benzo[*a*]pyrene: solid lines, 5  $\mu$ g; broken lines, 1  $\mu$ g. Spontaneous revertants have not been subtracted. Data from Edith Yamasaki.

TABLE 2<sup>a</sup>  
INDUCTION OF RAT LIVER ENZYMES

Induction method	S9 per plate ( $\mu$ l)	Revertants of TA1538 per plate <sup>b</sup>		
		Benzo[ <i>a</i> ]- pyrene (5 $\mu$ g)	3-Methylchol- anthrene (50 $\mu$ g)	2-Acetyl aminofluorene (50 $\mu$ g)
Aroclor 1254	10	127	37	750
	20	318	71	3320
	50	443	206	9600
	100	189	349	3420
	150	146	297	4280
Phenobarbital	10	—	85	1050
	20	—	136	3020
	50	45	256	13700
	100	23	356	14200
	150	17	207	12200
3-Methylcholanthrene (3-MC)	10	15	—	59
	20	121	—	620
	50	296	49	2045
	100	287	35	7187
	150	223	52	5640
Uninduced <sup>c</sup> (corn oil)	50	31	10	2344
	100	43	17	5824
	150	3	16	7184

Plate incorporation assays were performed as described in *Mutagenesis assays* using S9 samples which were prepared from 200-g rats according to the procedures in *Induction rat liver enzymes*.

<sup>a</sup> Ames et al., 1975b.

<sup>b</sup> The number of spontaneous revertants (< 30) has been subtracted from the appropriate experimental values. Assay plates were incubated for 2 days at 37° and then scored for revertants.

<sup>c</sup> The controls were 200-g rats which were injected with corn oil, the vehicle used for Aroclor 1254 and 3-methylcholanthrene induction. Induction with sodium phenobarbital (0.1% in the drinking water) and 3-MC (80 mg/kg, i.p.) was described previously (Ames et al., 1973b).

clogs the filter. It is preferable to filter by pressure rather than by vacuum to avoid foaming which can denature the S9 enzymes.

*Comparison of S9 preparations:* A quantitative comparison of different S9 preparations for activation of a carcinogen cannot be made using only one S9 concentration. As in any enzyme assay, the activity must be proportional to the amount of enzyme added in order to determine specific activity. In the case of S9 preparations, the enzyme activity is measured in terms of histidine revertants and must be proportional to the amount of S9 added (see Table 2). Thus a multi-point comparison is necessary. As can be seen from Fig. 1A, if one were to compare two S9 preparations for the activation of 2-aminofluorene using only one concentration of S9 (50  $\mu$ l/plate), the more active S9 preparation may actually appear to be less

active. It is also advisable to compare S9 preparations using several concentrations of mutagen.

*Stability of frozen S9 preparations.* We have not determined how long the microsomal enzymes remain active at  $-80^{\circ}$  but we have found no appreciable loss of activity for 2-aminofluorene or for benzo[*a*]pyrene in preparations that have been stored for 2 years at this temperature. At SRI International, S9 has been stored at  $-80^{\circ}$  for more than 3 years without loss of activity (Mortelmans, 1982). Ashwood-Smith (1977, 1980) has reported that long-term stability of frozen S9 preparations is achieved only at temperatures below  $-130^{\circ}$  at which temperature the addition of DMSO or other cryoprotective agents is not required. Unfortunately most laboratories do not have storage facilities at these extremely low temperatures and we feel that a more practical solution is to store S9 at  $-80^{\circ}$  in smaller batches that can be used within a year or two.

*Commercial sources of S9.* S9 preparations can be purchased from the AMC Cancer Research Center and Hospital, c/o Dr. Elias Balbinder, 6401 W. Colfax Ave., Lakewood, Colorado 80214; Microbiological Associates, c/o Dr. Steve Haworth, 5221 River Road, Bethesda, Maryland 20816; Litron Laboratories, 1351 Mt. Hope Ave., suite 207, Rochester, New York 14620; or from Litton Bionetics, c/o Dr. David Brusick, 5516 Nicholson Lane, Kensington, Maryland 20795. S9 is also available in Europe from Dr. E. Fresenius KG, Chem-Pharm. Industrie. Postfach 1809, D-6370 Oberursel, West Germany. Commercial S9 preparations should be tested for sterility and enzyme activity.

*S9 recommendations.* For optimum mutagenesis, the concentration of S9 per plate is critical, and can be variable from one compound to another. Too much S9 as well as too little can drastically lower the mutagenic response. We routinely test this variable to determine the optimum conditions for mutagenesis with a particular compound. We also test each new S9 preparation with several compounds such as benzo[*a*]pyrene, or 7,12-dimethylbenz[*a*]anthracene and an aromatic amine such as 2-acetylaminofluorene or 2-aminofluorene to find the optimum amount of S9 for general screening (see Fig. 1). We found that both benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene give a minimal response with uninduced S9 (Table 2; E. Yamasaki, unpublished data) which makes them useful as indicators of the efficiency of S9 induction. Zeiger et al. (1979) and Ong et al. (1980) had similar results with benzo[*a*]pyrene but found 2-aminoanthracene mutagenicity to be depressed with Aroclor treatment.

For general screening, we recommend a concentration of 20  $\mu$ l of S9 per plate. For this concentration, the S9 mix will contain 0.04 ml of S9 fraction per ml of mix. If a compound is negative using this concentration of S9, it should be retested with 50  $\mu$ l of S9 per plate. Ideally, a compound would be tested with both concentrations of S9 in the same assay but this would add considerably to the cost.

Other tissues and species have been used as a source of S9 (reviewed in Hollstein et al., 1979). There have been several reports in the literature that hamster liver S9 is more effective than rat liver S9 for activating several of the nitrosamines (Bartsch et al., 1975c; Prival et al., 1979; Matsushima et al., 1979; Raineri et al., 1981) and it has been suggested (Prival and Mitchell, 1981) that hamster liver S9 be used to test

any *N*-nitroso compound that is negative with rat liver S9.

**S9 mix.** The components of the standard S9 mix are 8 mM MgCl<sub>2</sub>, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate, pH 7.4, and S9 in a concentration of 0.04 ml per ml of mix. A higher concentration of S9 (0.1 ml of S9 per ml of mix) may be required for some test compounds. S9 mix is prepared fresh for each mutagenicity assay and may be kept on ice for several hours without loss of activity. See the *Recipe* section for the preparation of S9 mix and stock solutions of NADP, glucose-6-phosphate, phosphate buffer, and the MgCl<sub>2</sub>/KCl salts.

### Top agar

Top agar containing 0.6% Difco agar and 0.5% NaCl is autoclaved and stored at room temperature in volumes of 100 ml. We use rubber-lined plastic screw caps for the agar bottles to prevent evaporation on the shelf. Before use, the cap is loosened and the agar is melted by placing the bottle in a steam bath or microwave oven. 10 ml of a sterile solution of 0.5 mM L-histidine · HCl/0.5 mM biotin are added to the molten agar and mixed thoroughly by swirling. It makes no difference whether the histidine/biotin solution is filter-sterilized or autoclaved (Friederich et al., 1982). See *Recipe* section. Culture tubes (13 mm × 100 mm) fitted with plastic or metal caps (Bellco) are used to contain the 2-ml portions of top agar used in mutagenesis assays (see *Plate incorporation*).

The trace of histidine in the top agar allows all the bacteria on the plate to undergo several divisions and produces a faint background lawn which is visible to the naked eye and can be examined under a dissecting microscope. DNA replication is necessary in many cases for mutagenesis to occur and the background lawn is essential to the test as an indicator of inhibition of growth caused by the test chemical. This is discussed in *Interpretation of results*. A further increase in the amount of histidine on the plate enhances mutagenesis but also causes heavy growth of the background lawn that obscures the revertants. Accurate pipetting of the top agar into the culture tubes and consistent technique in pouring the agar overlay are essential to minimize differences in histidine content from one plate to the next.

### Agar plates

Plates for the mutagenicity assay contain 30 ml of minimal glucose agar medium. The medium is 1.5% Bacto-Difco agar and 2% glucose in Vogel-Bonner medium E (Vogel and Bonner, 1956). See the *Recipe* section. Sterile, disposable plastic petri plates (100 mm × 15 mm) are used. We use Falcon plates (No. 1029) that have been sterilized by  $\gamma$ -radiation. These plates are available from a number of commercial suppliers. Muta-assay plates have been discontinued.

## The mutagenicity test

**Plate incorporation test.** The plate incorporation test (Ames et al., 1973a, 1973b, 1975b) consists of combining the test compound, the bacterial tester strain, and S9 mix in soft agar which is poured onto a minimal agar plate. Positive and negative controls are also included in each assay. After incubation at 37° for 48 h, revertant colonies are counted.

In a modification of the plate incorporation procedure, a preincubation step precedes addition of the top agar. This modification is better for some compounds and appears to be at least as good for other compounds tested. See the *Preincubation* section.

All glassware, reagents, media and petri plates are sterile. See the *Recipe* section for preparation of stock solutions of reagents and media.

**Procedure:** Add histidine and biotin to the top agar according to the directions in the *Top agar* section. Distribute 2 ml of top agar into 13 × 100 mm capped culture tubes held at 45° in a heating block. Add 0.1 ml of a fresh overnight culture of the tester strain, 0.1 ml or less of the test chemical, and 0.5 ml of S9 mix. The compound is tested with and without S9 mix and both positive and negative control plates are included in the assay. Duplicate plates are poured for each dose of mutagen. Negative controls containing the bacteria, S9 mix and solvent (but no test chemical) are required to establish the number of colonies that arise spontaneously for each of the tester strains. This control is important because the S9 can influence the number of spontaneous revertants. Positive controls contain standard diagnostic mutagens specific for each tester strain (see the *Interpretation of results* section). The bacteria can remain at 45° for a few minutes without loss of viability but the S9 mix should not be left at this temperature for more than a few seconds. Mix the test components by vortexing the soft agar for 3 sec at low speed and then pour onto a minimal glucose agar plate. To achieve a uniform distribution of the top agar on the surface of the plate, quickly tilt and rotate the uncovered plate and then place it, covered, on a level surface to harden. The mixing, pouring, and distribution should take less than 20 sec and the plates should be left to harden for several minutes. It is important to follow these time limits. If the top agar starts to harden in mid-operation a stippled surface will result which makes scoring of revertants difficult. Belser et al. (1981) have reported that the greatest source of variability in the test results is nonuniformity of the soft agar thickness and they stress the importance of placing the freshly poured plates on surfaces that are absolutely level. The plates are covered promptly with brown paper or other suitable material to avoid the effects of light on photosensitive chemicals. Within an hour the plates should be inverted and placed in a dark, vented, 37° incubator. After 48 h the revertant colonies on the test plates and on the control plates are counted, and the presence of the background lawn on all plates is confirmed. A lawn that is thin compared to the lawn on the negative control plate is evidence of bacterial toxicity. Colonies appearing on a plate that has no background lawn are not revertants and should not be scored. These colonies arise from the surviving bacteria that live off the histidine present in the top agar. See the section on *Spontaneous reversion* for the number of colonies that typically arise

spontaneously for each of the tester strains.

*Spot test.* This is a variation of the plate incorporation test in which the mutagen is left out of the soft agar overlay and is applied directly to the surface of the minimal agar plate after it has been seeded with the bacterial tester strain and S9 mix. A few crystals of solid mutagens or approximately 10  $\mu$ l of liquid mutagens can be added to the agar surface. We have applied mutagens to sterile 6 mm filter paper discs but some chemicals such as daunomycin are detected much less efficiently on discs, possibly due to adsorption. Many water-insoluble compounds do not diffuse into the water-based agar and may give rise to false negatives. The plates can be left at room temperature for upto an hour before applying the mutagens but it is best to do this promptly after the top agar hardens.

### Positive controls (diagnostic mutagens)

In each experiment we routinely include positive mutagenesis controls using diagnostic mutagens to confirm the reversion properties and specificity of each strain and the efficacy of the S9 mix. The characteristic reversion patterns of the standard strains to some diagnostic mutagens are shown in Tables 3 and 4. Spot

TABLE 3

DIAGNOSTIC MUTAGENS: FOR CONFIRMING REVERSION PROPERTIES OF TESTER STRAINS IN SPOT TESTS

Mutagen	Amount spotted in 10 $\mu$ l	S9	TA97	TA98	TA100	TA102
Daunomycin <sup>a</sup>	5.0 $\mu$ g	-	-	+	-	++
Sodium azide	1.0 $\mu$ g	-	±	-	++++	-
ICR-191	1.0 $\mu$ g	-	++++	+	++	+
Mitomycin C	2.5 $\mu$ g	-	inh	inh	inh	+++
2,4,7-TNFone	0.1 $\mu$ g	-	++	++++	++	++
NPD	20.0 $\mu$ g	-	++	+++	+	+
NQNO	10.0 $\mu$ g	-	±	++	++++	+++
MMS	2.0 $\mu$ l	-	+	-	+++	++++
Dexon	50.0 $\mu$ g	-	++++	+++	++	+++
2-AF	20.0 $\mu$ g	+	++	++++	+++	+

Symbols for the number of revertants/plate (spontaneous subtracted): - = < 20; + = 20-100; ++ = 100-200; +++ = 200-500; ++++ = > 500. Daunomycin and sodium azide were dissolved in H<sub>2</sub>O. All other compounds were dissolved in DMSO. PCB-induced rat liver S9 was used in a concentration of 20  $\mu$ l/plate for the activation of 2-AF.

<sup>a</sup> Produces a minimal response in the spot test; should be tested by plate incorporation (see Table 4).

*Abbreviations:* ICR-191, 2-methoxy-6-chloro-9-(3-(2-chloroethyl)aminopropylamino)acridine · 2HCl; 2,4,7-TNFone, 2,4,7-trinitro-9-fluorenone; NPD, 4-nitro-*o*-phenylenediamine; NQNO, 4-nitroquinoline-*N*-oxide; MMS, methyl methanesulfonate; 2-AF, 2-aminofluorene; inh, inhibition of growth due to toxicity of the mutagen.

tests with these mutagens are generally adequate for the qualitative assessment of the sensitivity of the tester strains. Zeiger et al. (1981) suggest the use of filter paper discs impregnated with the diagnostic mutagens as a convenient and rapid scheme for confirming the sensitivity and specificity of the strains. When stored frozen in a desiccator jar, the discs are stable for 2 years (Zeiger, 1982). If the sensitivity of a strain is in doubt, quantitative reversion values can be determined by incorporating the mutagens into the top agar and counting revertant colonies. A single dose of the appropriate mutagen is used as a positive control for each strain. In the case of MNNG and 9-aminoacridine, two of the diagnostic mutagens previously recommended (Ames et al., 1975b), erratic reversion rates are frequently observed with TA1535 and TA1537, respectively, because the dose-response effects are non-linear. As a consequence, it is difficult to obtain reproducible results with a single dose of MNNG or 9-aminoacridine. For this reason, and because MNNG is extremely hazardous, we no longer use these two diagnostic mutagens. Sodium azide replaces MNNG as the positive control for TA1535 and TA100. Sodium azide is stable in water and it does not have to be prepared daily. It is almost as active on TA1535 as it is on TA100 but the elimination of TA1535 from the recommended tester set

TABLE 4  
REVERSION OF TESTER STRAINS

Mutagen	Amount	S9	TA97	TA98	TA100	TA102 <sup>a</sup>
Daunomycin	6.0 µg	—	124	3123	47	592
Sodium azide	1.5 µg	—	76	3	3000	188
ICR-191	1.0 µg	—	1640	63	185	0
Streptonigrin	0.25 µg	—	inh <sup>b</sup>	inh <sup>b</sup>	inh <sup>b</sup>	2230
Mitomycin C	0.5 µg	—	inh <sup>c</sup>	inh <sup>c</sup>	inh <sup>c</sup>	2772
2,4,7-TNFone	0.20 µg	—	8377	8244	400	16
NPD	20 µg	—	2160	1599	798	0
NQNO	0.5 µg	—	528	292	4220	287
MMS	1.0 µl	—	174	23	2730	6586
Dexon <sup>d</sup>	50 µg	—	2688	1198	183	895
2-AF	10 µg	+	1742	6194	3026	261
B(a)P	1.0 µg	+	337	143	937	255

The numbers represent His<sup>+</sup> revertants taken from the linear portion of dose-response curves. Control values were subtracted. PCB-induced rat liver S9 (20 µl/plate) was used for activation of 2-AF and B(a)P.

<sup>a</sup> Unpublished data from David Levin.

<sup>b</sup> No mutagenicity detected in the non-toxic range (< 0.25 µg); < 70 revertants/0.005 µg of streptonigrin on TA100.

<sup>c</sup> Mitomycin C is lethal on ΔuvrB strains (Levin et al., 1982b).

<sup>d</sup> Data from Levin et al. (1982a).

*Abbreviations:* ICR-191, 2-methoxy-6-chloro-9-(3-(2-chloroethyl)aminopropylamino)acridine · 2HCl; 2,4,7-TNFone, 2,4,7-trinitro-9-fluorenone; NPD, 4-nitro-*o*-phenylenediamine; NQNO, 4-nitroquinoline-*N*-oxide; MMS, methyl methanesulfonate; 2-AF, 2-aminofluorene; B(a)P, benzo[*a*]pyrene; inh, inhibition of bacterial growth due to toxicity of the mutagen.

makes this mutagen diagnostic for TA100. If necessary, TA100 can be distinguished from TA1535 by the R-factor-requiring mutagen, methyl methanesulfonate or nitrofurantoin. We have replaced 9-aminoacridine with ICR-191 which is diagnostic for TA97 at a concentration of 1  $\mu\text{g}$  per plate (see Tables 3 and 4). ICR-191 gives a slight response with TA100 but it is approximately 10 times more active on TA97 in the plate incorporation test. Positive controls using chemicals requiring metabolic activation confirm that the S9 is active. For this purpose 2-aminofluorene (2  $\mu\text{g}$ ) can be spot-tested but the polycyclic hydrocarbons, such as benzo[a]pyrene which do not diffuse in the agar must be incorporated directly into the agar overlay.

## Interpretation of results

*Plate incorporation test.* The plate incorporation test in which the mutagen, bacteria, and S9 mix are added directly to the top agar, is the standard method that has been used for validating the test using hundreds of chemicals. For initial screening of a chemical we recommend testing concentrations over a three-log dose range in the presence and absence of the standard S9 mix. See the *S9 recommendations* section. A positive or questionable result should be confirmed by demonstrating a dose-response relationship using a narrower range of concentrations. Compounds that are negative can be retested using the preincubation procedure (see *Modifications of the mutagenicity test*). For most mutagens we have tested, there is a concentration range that produces a linear dose-response curve. The number of revertants per plate reported for a mutagen should be taken from this region of the curve. Occasionally, non-linear dose-response curves are obtained, as in the case of 9-aminoacridine, MNNG, diethylsulfate and ethylmethanesulfonate (McCann et al., 1975b). Nonlinearity should be indicated in reporting mutagenicity data. Most mutagens are toxic to the bacteria at some concentration. In the toxic range there is a decrease in the number of revertants on the plate. The dose-response curve for 2,4,7-trinitro-9-fluorenone (2,4,7-TNFone) with TA97 illustrates this toxic effect (Fig. 2). If one were to test 2,4,7-TNFone at only one concentration which happens to be in the descending portion of the curve, the results would be misleading as to the quantitative mutagenic activity of this compound.

Routine examination of the bacterial background lawn resulting from the trace of histidine added to the top agar (see the *Top agar* section) is an aid in determining the toxicity of the test chemical and is essential to the interpretation of results. If massive cell death has occurred, the background lawn on the test plates will be sparse compared to control plates. In this case more histidine is available to the surviving bacteria which will undergo more cell divisions and will appear as small colonies. These colonies can be mistaken for revertants if one has failed to observe the absence of a normal background lawn. The his<sup>+</sup> character of these colonies can be verified by testing for growth on minimal glucose agar containing biotin but no histidine.

As indicated in the section, *Spontaneous reversion*, the number of spontaneous revertants per plate for a particular tester strain depends on the number of histidine

auxotrophs in the lawn after the 48-h incubation. This is determined by the concentration of histidine in the agar and is independent of the number of bacteria plated, within the limits specified in the *Spontaneous reversion* section. On the other

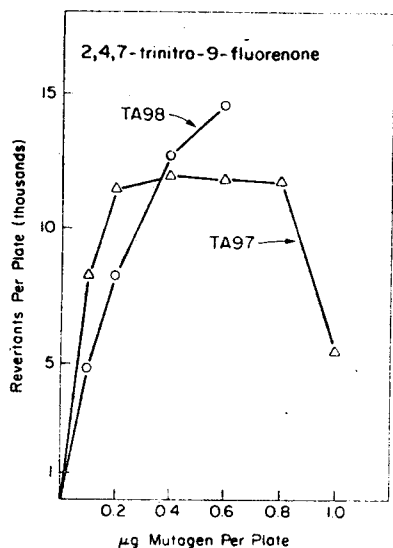


Fig. 2. Dose-response effects of 2,4,7-trinitro-9-fluorenone with TA97 and TA98. Spontaneous revertants have been subtracted.

hand, the number of induced revertants per plate does depend on the number of bacteria plated. Because mutational events are rare, it is essential to use large populations of bacteria in mutagenicity testing (Ames, 1971). Maximum sensitivity is achieved by plating  $2 \times 10^8$  cells (Green and Muriel, 1976). If fewer cells are plated, the number of spontaneous revertants will be normal but the number of induced revertants can be low for a particular concentration of mutagen.

It is invalid to calculate the induced mutation frequency based on the number of viable cells plated after treatment with a test compound. Green and Muriel (1976) have shown that the frequently misused equation

$$\text{Mutation frequency} = \frac{\text{mutants/test plate}}{\text{viable cells/test plate}} - \frac{\text{mutants/control plate}}{\text{viable cells/control plate}}$$

leads to anomalous test results. They demonstrated that the calculation of mutation frequency using this equation can show distilled water (or any substance that kills bacteria) to be mutagenic. The expression of mutagenicity data in terms of mutation frequency requires knowledge of the number of bacterial survivors on the plate after the 48-h incubation. The Salmonella assay does not easily lend itself to the determination of plate survivors and the test is designed to circumvent the problem by adjusting the mutagen doses to a range that produces a linear response below the toxic range. Mutagenicity test results can be reported as revertants per  $\mu\text{g}$  of test compound taken from the linear portion of the dose-response curves.

A quantitative measurement of toxicity is not part of the standard assay. A recent modification of the test which deals with this problem is discussed in *Modifications of the mutagenicity test*.

Although the standard amount of S9 recommended for general testing should permit detection of a wide variety of chemical mutagens requiring metabolic activation, it is advisable to determine the optimum concentration of S9 in the mix for activation. This varies with the type of compound and the concentration tested. See *S9 induction* section.

Sterility controls for the S9 mix and the test chemicals are also routinely included in each experiment. Sterility of test chemicals dissolved in DMSO is rarely a problem but aqueous solutions may require filter sterilization.

*Spot test.* The spot test is the simplest way to test compounds for mutagenicity and is useful for the initial rapid screening of large numbers of compounds. We have tested 169 different hair dyes for mutagenicity using this method (Ames et al., 1975a). We have also used it in student laboratory experiments. The spot test has several advantages. A few crystals of a solid mutagen or  $\mu\text{l}$  of a liquid mutagen can be put directly on the agar surface, thus eliminating the time-consuming preparation of solutions of the chemicals to be tested. As the test compound diffuses out from the central spot, a range of concentrations is tested simultaneously. The spot test indicates whether the S9 enzymes are required for mutagenesis, and it also gives a preliminary indication of the toxicity of the chemical by the size of the zone of inhibition of the bacterial background lawn around the spot. It should be emphasized, however, that the size of the zone is also a function of the chemical's ability to diffuse through the agar and it is by no means a quantitative indication of toxicity. In the case of a positive result, the spot test indicates which tester strain should be used to demonstrate the dose-response relationship. The method can be used to test samples that are not sterile, provided the colonies directly under the spot are not counted. Because of these advantages, it may be useful to test new compounds by the spot test before doing the standard plate incorporation test.

Although the spot test is useful, it is primarily a qualitative test and has distinct limitations. It can be used only for testing chemicals that are diffusible in the agar, and therefore most polycyclic hydrocarbons and other water-insoluble chemicals are not easily detected by this procedure. It is much less sensitive than the standard plate incorporation test as only relatively few bacteria on the plate are exposed to the chemical at any particular dose level. Also, the concentration of mutagen at the perimeter of the zone of inhibition may not always be optimal for mutagenesis, as in the case of nitrofurantoin (commercial discs) which produces toxic zones but no revertant colonies (Zeiger et al., 1981). Therefore, a negative result in the spot test alone is not a sufficient indication of non-mutagenicity in *Salmonella*.

A positive result in a spot test is generally not considered to be adequate evidence for mutagenicity. Mutagenicity should be confirmed by demonstrating a dose-response relationship using the standard plate incorporation test.

## Statistical analysis of test data

A number of statistical approaches to the analysis of test data have appeared in the literature (Weinstein and Lewinson, 1978; Katz, 1979; Venitt and Crofton-Sleigh, 1979; Stead et al., 1981). Margolin et al. (1981) have criticized these authors for adopting the Poisson model for the distribution of revertants per plate, for ignoring the multigenerational aspects of the test and for disregarding toxicity or treating it in an unsystematic way. Margolin et al. (1981) have constructed a family of statistical models for data analysis that do not presuppose or exclude Poisson variability. Their models consider mutation and toxicity as competing risks. Bernstein et al. (1982) take an empirical approach based on the assumption that the initial portion of the dose-response curve is linear, and they describe a procedure for deciding which points are on the linear portion of the curve.

## Modifications of the mutagenicity test

*Urine assay.* Durston and Ames (1974) and Commoner et al. (1974) have reported procedures for the detection of mutagenic metabolites of 2-acetylaminofluorene in urine. The addition of commercial  $\beta$ -glucuronidase to the top agar along with the urine, S9 mix, and bacteria allows detection of metabolites of these carcinogens which are excreted in urine primarily as  $\beta$ -glucuronide conjugates. By this method mutagenic activity is readily demonstrated in the urine of rats administered as little as 200  $\mu\text{g}$  (1.6 mg/kg) of 2-acetylaminofluorene. We have therefore recommended routine mutagenicity testing of the urine of animals administered a chemical for toxicological studies (Durston and Ames, 1974).

A problem that may arise in the analysis of urinary metabolites present in low concentrations is the concomitant presence in urine of histidine in the range of 0.7–1.3  $\mu\text{mole/ml}$ . When it is necessary to assay a large volume of urine in the *Salmonella* test, the additional urinary histidine causes an enhanced growth of the bacterial lawn. This overgrowth of the bacteria obscures the presence of revertant colonies and interferes with scoring. In addition, the number of spontaneous revertants that arise because of the additional histidine may give a false positive result. The amount of urine that can be assayed directly is therefore limited to rather small volumes. To circumvent this limitation, Yamasaki and Ames (1977) used an adsorptive process with XAD-2 resin, a styrene divinylbenzene copolymer, which allows the concentration of relatively nonpolar mutagenic components. Histidine, a polar compound, is not efficiently adsorbed on XAD-2 and thus its effect on the *Salmonella* assay is minimized. This technique has been used to demonstrate the presence of mutagens in the urine of cigarette smokers (Yamasaki and Ames, 1977). Aeschbacher and Ruch (1982) investigated the inhibitory effect of the urine on mutagenicity. The sensitivity of the XAD-2 method for testing urine has recently been improved by mixing a concentrated bacterial suspension with urine extract and liver homogenate in a 90-min preincubation (Kado et al., 1982).

*Desiccator assay.* The testing of volatile, relatively water-insoluble compounds,

whether gas or liquid, cannot be done quantitatively in the manner described for plate incorporation of test chemicals in solution. By a modification of the plate test, Rannug et al. (1974) were able to demonstrate the mutagenicity of the gas, vinyl chloride, by exposing petri plates seeded with TA1535 (+S9 mix) to known vinyl chloride/air mixtures in a 10-l desiccator for various lengths of time. Bartsch et al. (1975a) also tested vinyl chloride using this procedure and later showed the method applicable to the testing of vinylidene chloride and 2-chlorobutadiene (1975b). Simmon et al. (1977) and Simmon (1981) have reported that many alkyl halides are mutagenic when assayed in desiccators. This class of compounds is not detected or is weakly detected in the standard Salmonella assay. Among the alkyl halides found to be mutagenic with this procedure are methylene chloride, epichlorhydrin, and dichloroethane. Simmon (1981) found the desiccator technique to be more sensitive than the standard test for most alkyl halides, even when the boiling point exceeds 175°, which implies that it is not for use exclusively with volatile, low molecular weight compounds. Bridges (1978) has increased the sensitivity of the desiccator assay by spreading the bacteria on the surface of the agar.

*Preincubation test.* Some mutagens, such as dimethyl- and diethylnitrosamine are poorly detected in the standard plate incorporation assay and should be tested using a modification of the standard procedure. The most widely used test modification is the preincubation assay first described by Yahagi et al. (1975) in which carcinogenic azo dyes were found to be mutagenic. They incubated the mutagen, S9 mix, and bacteria for 20–30 min at 37°C and then added the top agar. The preincubation assay has also been used to detect the mutagenicity of 10 carcinogenic nitrosamines (Yahagi et al., 1977) and several carcinogenic alkaloids (Yamanaka et al., 1979). Rosenkranz et al. (1980b) demonstrated the mutagenicity of some labile chemicals and a series of volatile chemicals using the preincubation assay.

The mutagenic activity of aflatoxin B<sub>1</sub>, benzidine, benzo[*a*]pyrene, and methyl methanesulfonate has been determined using both plate incorporation and preincubation procedures and in all cases the preincubation assay is of equal or greater sensitivity than the plate incorporation assay (Matsushima et al., 1980). The increased activity is attributed to the fact that the test compound, S9, and bacteria are incubated at higher concentrations in the preincubation assay than in the standard plate incorporation test (Prival et al., 1979). The procedure described below is based on the recommendations of Matsushima et al. (1980).

*Preincubation procedure:* Deliver 0.1 ml of test solution (dissolved in H<sub>2</sub>O or DMSO) to sterile 13 × 100 mm capped culture tubes placed in an ice bath. Add 0.1 ml of overnight culture of the tester strain and 0.5 ml of S9 mix or 0.5 ml of a control containing buffer but no S9. Vortex the tube gently and incubate at 37°C for 20 min or at 30° for 30 min. The choice of conditions is purely a matter of convenience according to the size of the experiment and whether one or more persons are performing the experiment. These two sets of conditions give comparable results (Matsushima et al., 1980). The tubes are shaken at moderate speed during the incubation. A Thermolyne Dri-Bath attached to a model G2 New Brunswick laboratory rotator makes a convenient incubation apparatus which can be placed in the fume hood. The Dri-Bath is fitted with aluminum blocks, with 20 wells each, for 13-mm tubes.

The S9 mix used in our standard plate incorporation assay can be used for the reincubation procedure. Some laboratories use additional cofactors in the S9 mix (Nagao et al., 1977; Yamanaka et al., 1979) and substitute NADPH for the IADPH-generating system. There appears to be no particular advantage in using IADPH, but it is used extensively in Japan because it is inexpensive there. In general, the same concentrations of S9 recommended for the standard plate assay are used in the preincubation procedure. The optimum concentration of S9 can be determined by testing a constant dose of test compound with increasing concentrations of S9.

If the test compounds are not soluble in DMSO or water, alternative solvents can be used (Maron et al., 1981). With any organic solvent, it is advisable to keep the volume at a minimum to avoid killing the bacteria or destroying the S9 enzymes. This is crucial in the preincubation assay where the total volume of the incubation mix is small relative to the solvent component. See the following section on solvents.

The preincubation modification can be used routinely or when inconclusive results are obtained in the standard plate incorporation assay. The preincubation assay requires an extra step and therefore involves more work than the standard test but many laboratories use it routinely because of the increased sensitivity for some compounds. Its use in screening assays has been recommended by De Serres and Shelby (1979).

*Solvents compatible with the Salmonella test.* Compounds to be tested for mutagenicity are routinely dissolved in DMSO if they are insoluble in water. Chemicals that are also insoluble in DMSO must be dissolved in alternative solvents. 14 organic solvents were screened for compatibility with the Salmonella test and 12 were found to be satisfactory under the conditions specified (Maron et al., 1981). These solvents are dimethyl sulfoxide, glycerol formal, dimethyl formamide, formamide, acetonitrile, 95% ethanol, acetone, ethylene glycol dimethyl ether, 1-methyl-2-pyrrolidinone, *p*-dioxane, tetrahydrofurfuryl alcohol, and tetrahydrofuran. The maximum amounts of these solvents that are compatible with the Salmonella test were determined. *p*-Dioxane should be used with caution as it is a carcinogen.

*Toxicity assay.* Since the criterion of mutagenicity is the dose-response relationship, there must be a range in which the number of induced revertants increases with the dose. Most mutagens are also toxic to some extent, and consequently the number of revertants usually decreases at higher concentrations. This is not a problem with most strongly positive mutagens such as benzo[*a*]pyrene or aflatoxin B<sub>1</sub> which show a linear dose-response relationship with the tester strains at concentrations below the range of strong toxicity. When the test compound is weakly mutagenic and strongly toxic, however, it may be difficult to find a range of concentrations in which the mutagenic potential is not masked by toxicity.

Recently, Waleh et al. (1982) have developed an ingenious assay for measuring toxicity quantitatively. Their assay can be used in conjunction with the standard Salmonella mutagenicity test in special cases where the test chemicals are highly toxic. It is based on the use of two sets of strains that are isogenic with the standard tester strains except for the His character. One set of strains is His<sup>+</sup> and the other has a Tn10-generated His<sup>-</sup> mutation. These His<sup>-</sup> double mutants, called 'filler cells',

are unable to revert to histidine independence and are used to mimic the background lawn present in the standard mutagenicity assay. The killing of His<sup>+</sup> cells by the test compound is measured under conditions that are nearly identical to those of the standard assay. Approximately 500 His<sup>+</sup> cells and 10<sup>8</sup> His<sup>-</sup> cells are exposed to the test chemical. Survival of the His<sup>+</sup> cells in the presence of the His<sup>-</sup> 'filler cells' is used to determine the toxicity of the test compound.

*Detection of mutagenic glycosides with fecal extract.* Many of the plants eaten by animals and by man contain mutagenic substances that exist in an inactive, conjugated form as glycosides. The mutagenic aglycones are released by the action of a wide variety of glycosidases present in the bacteria that live in the human gut. The mutagenic glycosides are negative in the Salmonella test because they are not hydrolyzed by the liver S9 enzymes. In our laboratory, Tamura et al. (1980) used fecalase, an enzyme preparation from human feces, as a model for activation of dietary glycosides to mutagens by intestinal bacteria. The activity of fecalase was shown to be attributable to various glycosidases that were effective in hydrolyzing many naturally occurring glycosides to mutagens in the Salmonella test. Fecalase can be stored at -80° in the same manner as S9. Brown and Dietrich (1979) used a rat cecal extract in the Salmonella assay as a source of glycosidase activity, and Matsushima et al. (1979) demonstrated the mutagenicity of the carcinogenic glycoside, cycasin, by preincubation with almond  $\beta$ -glucosidase and hisG46. Mixed glycosidases from marine molluscs,  $\beta$ -glucosidase from almonds, and  $\beta$ -glucuronidase from *Prunus amygdalus* are available commercially (see Brown and Dietrich, 1979).

*Cofactors for detecting azo dyes.* The mutagenic potential of azo dyes is of interest because they are widely used to color foods and cosmetics. Azo compounds can be reduced by anaerobic bacteria in the gut to form aromatic amines (Gingell and Walker, 1971) many of which are carcinogens. Yahagi et al. (1975) detected mutagenicity in *N,N*-dimethyl-4-aminoazobenzene (DAB) and *N*-methyl-4-aminoazobenzene (MAB) using the preincubation procedure. Sugimura et al. (1977) found that the addition of riboflavin to the S9 mix enhanced the mutagenicity of some azo dyes. In their studies, riboflavin was obligatory for reversion of the tester strains by some azo compounds, such as DAB, but had no effect when used with Red dye No. 2 which was not mutagenic with or without riboflavin. Zeiger and Pagano (1982) found DAB to be mutagenic in the standard plate incorporation procedure, using two concentrations of S9 in the standard S9 mix. Prival and Mitchell (1982) modified Sugimura's protocol by using flavin mononucleotide (FMN) rather than riboflavin for the reduction of azo bonds in benzidine dyes and by substituting uninduced hamster liver S9 for Aroclor 1254-induced rat liver S9. The concentration of S9 was increased to 150  $\mu$ l per plate and the cofactor mix included exogenous glucose 6-phosphate dehydrogenase, NADPH, and 4 times the standard amount of glucose 6-phosphate. A 30-min preincubation preceded addition of the top agar. All of the changes described were essential for optimum expression of mutagenicity of the azo dyes derived from the mutagenic aromatic amines, benzidine, *o*-tolidine, or *o*-dianisidine.

*Liver sulfotransferase-PAPS activation system.* Watabe et al. (1982) have re-

ported evidence for the activation of *N*-hydroxymethyl aromatics to mutagenic sulfate esters in rat liver cytosol in the presence of a 3'-phosphoadenosine-5'-phosphosulfate (PAPS) generating system. The PAPS-generating system consists of ATP, sodium sulfate and magnesium chloride. 7-Hydroxymethylbenz[*a*]anthracene (7-HMBA), a carcinogenic metabolite of 7,12-dimethylbenz[*a*]anthracene (DMBA), has no intrinsic mutagenicity to *Salmonella* but it can be activated by this modified S9 system to the reactive form, 7-HMBA sulfate, which is mutagenic to TA98. The mutagenicity of 7-HMBA in the presence of liver sulfotransferase is much greater than that of 7-HMBA (or DMBA) in the presence of liver monooxygenase. Some other sulfate esters of arylmethanols such as benzyl alcohol, 1- and 2-hydroxymethylnaphthalene, and 1-hydroxymethylpyrene were also mutagenic to TA98 in this system. It seems likely that S9 mix with the addition of PAPS or the PAPS generating system will be a useful adjunct to the standard test.

*Accelerated growth of the His<sup>+</sup> revertants.* This was achieved by enriching the base agar with amino acids other than histidine (Arimoto et al., 1981). Colonies were visible after a 24-h incubation at 37° when the following amino acids were added to the minimal glucose agar: glycine, alanine, leucine, valine, isoleucine, serine, threonine, methionine, proline, lysine, arginine, glutamic acid, phenylalanine, tyrosine and tryptophan.

### Mutagenicity information

The Environmental Mutagen Information Center (EMIC) provides mutagenicity information, without charge, for any chemical tested in the *Salmonella*/microsome assay or in any other mutagenicity tests that have been reported in the literature. For information, contact Mr. John Wassom, EMIC, Oak Ridge National Laboratories, Oak Ridge, Tennessee 37830. Phone: (615) 574-7871.

### Disposal of carcinogen and mutagen waste

We recommend using disposable test tubes, petri plates and micro-pipettes with disposable tips in order to avoid recirculation of these items with the rest of the laboratory glassware. The contaminated glassware is placed in cardboard boxes lined with two heavy-duty plastic bags. The bags and the boxes are sealed with strapping tape and labeled with the contents. Disposal of mutagen waste is by contrast with a firm that disposes of radioactive waste from the laboratory, in accordance with the regulations established by the Department of Environmental Health and Safety of the University of California.

### Safety precautions

*Handling mutagens carcinogens.* In our laboratory, every effort is made to isolate the areas where mutagens are used in order to avoid contamination of the labora-

tory. Mutagenicity assays are performed in a well-ventilated fume hood designated solely for this purpose. Class II type B hoods also provide a sterile work area. Standards for airflow in laboratory hoods used for mutagenicity assays have been set by the National Cancer Institute. They specify that open-faced hoods should have an average linear face velocity of 100 feet per min. Solids and volatile liquids used to prepare test solutions are also handled in the mutagen hood, and all weighings are done by weight difference to avoid opening the containers of solid or liquid test material in the laboratory outside of the hood. The investigator wears disposable plastic or latex gloves. Unfortunately, most gloves are not an absolute protection against mutagens dissolved in organic solvents and should be removed immediately and discarded if they are contaminated with a mutagen solution. In a test of 11 types of work gloves by the National Institute of Occupational Safety and Health, Weeks and McLeod (1980, 1982) found that all but one of the gloves tested with PCBs soaked through in less than 3 min. They found Viton to be the best glove material for use with solvents.

Petri plates containing test material are incubated in a 37° incubator reserved for mutagenesis work. The incubator is placed adjacent to the hood. A 2-inch flexible vacuum cleaner tube attached to the incubator outlet pipe connects to a low exhaust fan mounted on the outside wall of the hood. When the incubator door is opened, a micro-switch turns on the fan creating a differential pressure which moves the air out of the incubator into the hood. Petri plates containing volatile solvents should be placed in desiccators or in Dri-jars with one dose level per container.

*Handling the Salmonella strains.* *Salmonella typhimurium* can cause diarrhea and food poisoning, if ingested. *S. typhimurium* LT2, is the parent of all our tester strains. This strain is not very virulent and is used by geneticists all over the world. The standard tester strains are relatively harmless because the deep rough mutation (*rfa*) lowers virulence by orders of magnitude. The *gal*<sup>-</sup> strains (see Table 1) are also relatively non-virulent because they lack the galactose operon which is essential for making the lipopolysaccharide coating required for virulence (Ames, 1971). The pKM101 plasmid in strains TA98, TA100, TA97, and TA102, with one antibiotic resistance marker for ampicillin, should be a minimal hazard. Plasmids are extremely common in the enteric population in nature and most of the RTF plasmids isolated from *Salmonella* in hospitals contain many antibiotic resistance genes. Nevertheless, as a routine precaution with any *Salmonella* strain, we use plugged pipettes and autoclave any material containing *Salmonella* before it is washed or discarded. Laboratory benches are swabbed routinely with strong detergent and then with 70% alcohol. Material containing both carcinogens and *Salmonella* is discarded as described previously without autoclaving. It is a laboratory rule not to keep food in refrigerators containing carcinogens or *Salmonella*, and laboratory workers are not permitted to eat in the laboratory. It may be prudent to keep *Salmonella* away from mouse colonies.

## Chemical sources

**Mutagens and PCB.** Streptonigrin: Developmental Therapeutics Program, National Cancer Institute (not available commercially); sodium azide, daunomycin, 4-nitroquinoline *N*-oxide: Sigma; ICR-191: Terochem Laboratories, 5729 92nd Street, Edmonton, Alberta, Canada; 2-aminofluorene, benzo[*a*]pyrene, methyl methanesulfonate, mitomycin C, 2,4,7-trinitro-9-fluorenone, 4-nitro-*o*-phenylenediamine: Aldrich; Dexon, Aroclor 1254: Analabs, 80 Republic Drive, North Haven, Connecticut 06473. Aroclor is no longer available from Monsanto.

**Reagents, enzyme preparations, and nutrient broth.** Ampicillin trihydrate, tetracycline: Sigma; dimethylsulfoxide (spectrophotometric grade, in 100-ml bottles): Schwarz/Mann; D-biotin and L-histidine·HCl (monohydrate): ICN Nutritional Biochemicals; D-glucose-6-phosphate (monosodium salt), NADP (sodium salt): Sigma; sodium phenobarbital: Mallinckrodt; bacto yeast extract, bacto tryptone, skim milk: Difco; Oxoid nutrient broth No. 2: Oxoid USA Inc., 9017 Red Branch Road, Columbia, Maryland 21045 or K.C. Biological, P.O. Box 5441, Lenexa, Kansas 66215. Also available in Canada from Oxoid Canada Ltd., 145 Bentley Ave., Ottawa K2E 6T7. Liver S9: see *Commercial sources of S9*. XAD-2: Applied Science Division (formerly Applied Science Laboratories), P.O. Box 440, State College, Pennsylvania 16801.

## Equipment and supplies

Most of the equipment and supplies listed below can be obtained from several commercial suppliers. Common laboratory items such as flasks and glass pipettes are not included. Items followed by \* are used in the preparation of S9 and are not required if S9 is obtained from commercial sources.

**Large equipment:** Stereozoom microscope and microscope illuminator, automatic colony counter, manual colony counter, gyrorotary incubator, stationary incubator, laminar flow hood, small rotary shaker (to fit inside the hood) for use in preincubation assays. Refrigerated centrifuge\*, extra high torque stirrer\*, -80° freezer.

**Small equipment:** Adjustable micropipettes with disposable tips, 1 dram (4 ml) glass vials (for mutagen solutions) with teflon lined caps, disposable glass culture tubes (13 mm × 100 mm), constant temperature block with 72 holes for 13-mm tubes, heating blocks to hold one bottle of top agar and aluminum block inserts (with holes for 13-mm tubes), latex surgeon's gloves, sterile 1/4 inch filter paper discs for testing crystal violet sensitivity and for spot tests, plastic cryotubes for storage of S9, racks for cryotubes, sealed filtration units (0.2 μ pore size) for filter sterilization of stock solutions, vortexer, hand programmable hand calculator for correcting colony counts from calibrated automatic colony counters, 12-inch wooden applicator sticks for inoculating cultures, tissue grinder (piston-type teflon pestle with stainless steel rod and grinding vessel of borosilicate glass)\*, vacule ampules with long necks for freeze-drying cultures and sealing under vacuum, two-way oxygen gas torch for sealing ampules.

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## Recipes for stock solutions and media

Vogel-Bonner medium E (50X)

Use: Minimal agar

<i>Ingredient</i>	<i>Per liter</i>
Warm distilled H <sub>2</sub> O (45°C)	670 ml
Magnesium sulfate (MgSO <sub>4</sub> · 7 H <sub>2</sub> O)	10 g
Citric acid monohydrate	100 g
Potassium phosphate, dibasic (anhydrous) (K <sub>2</sub> HPO <sub>4</sub> )	500 g
Sodium ammonium phosphate (NaH <sub>2</sub> NH <sub>4</sub> (PO <sub>4</sub> · 4 H <sub>2</sub> O)	175 g

Add salts in the order indicated to warm water in a 2-liter beaker or flask placed on a magnetic stirring hot plate. Allow each salt to dissolve completely before adding the next. Adjust the volume to 1 liter. Distribute into two 1-liter glass bottles. Autoclave, loosely capped, for 20 min at 121°. When the solutions have cooled, tighten the caps.

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### 0.5 mM histidine/biotin solution

Use: Mutagenicity assay (add 10 ml to 100 ml of top agar)

<i>Ingredient</i>	<i>Per 250 ml</i>
D-Biotin (F.W. 247.3)	30.9 mg
L-Histidine · HCl (F.W. 191.7)	24.0 mg
Distilled H <sub>2</sub> O	250 ml

Dissolve the biotin by heating the water to the boiling point. This can be done in a microwave oven. Sterilize by filtration through a 0.22- $\mu$ m membrane filter or autoclave 20 min at 121°. Store in a glass bottle at 4°.

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## Top agar

Use: Mutagenicity assay

<i>Ingredient</i>	<i>Per liter</i>
Agar	6 g
Sodium chloride (NaCl)	5 g
Distilled H <sub>2</sub> O	1000 ml

The agar may be dissolved in a steam bath or microwave oven, or by autoclaving briefly. Mix thoroughly and transfer 100-ml aliquots to 250-ml glass bottles with screw caps. Autoclave for 20 min with loosened caps. Slow exhaust. Cool the agar and tighten caps.

Salt solution (1.65 M KCl + 0.4 M MgCl<sub>2</sub>)

Use: S9 mix for mutagenicity assay

<i>Ingredient</i>	<i>Per 500 ml</i>
Potassium chloride (KCl)	61.5 g
Magnesium chloride (MgCl <sub>2</sub> · 6 H <sub>2</sub> O)	40.7 g
Distilled H <sub>2</sub> O	to final volume of 500 ml

Dissolve ingredients in water. Autoclave for 20 min at 121°. Store in glass bottles in the refrigerator or at room temperature.

## 0.2 M sodium phosphate buffer, pH 7.4

Use: S9 mix for mutagenicity assay

<i>Ingredient</i>	<i>Per 500 ml</i>
0.2 M sodium dihydrogen phosphate (NaH <sub>2</sub> PO <sub>4</sub> · H <sub>2</sub> O) (13.8 g/500 ml)	60 ml *
0.2 M disodium hydrogen phosphate (Na <sub>2</sub> HPO <sub>4</sub> ) (14.2 g/500 ml)	440 ml *

\* These are approximate values. Test the pH. If it is too low, add more 0.2 M disodium hydrogen phosphate to pH 7.4. Sterilize by autoclaving for 20 min at 121°.

## 1 M NADP solution (nicotine adenine dinucleotide phosphate)

Use: S9 for mutagenicity assay

<i>Ingredient</i>	<i>Per 5 ml</i>
NADP (F.W. 765.4)	383 mg *
Sterile distilled H <sub>2</sub> O	5 ml

Add NADP to pre-weighed sterile glass tubes with screw caps. Do not add water. It is convenient to prepare a dozen or more of these dry aliquots at one time. Wrap

the tubes with metal foil to protect against light and label each tube with the correct weight. It is not necessary to weigh exactly 383 mg as long as the weight is indicated on the label along with the calculated volume of water to give a 0.1 M solution. Place all the tubes of weighed NADP in a jar with a tight fitting lid. Silica gel or other desiccant should be placed in the bottom of the jar. Store in a  $-20^{\circ}$  freezer. When needed for making S9 mix, remove one tube from the jar, add the specified amount of water and mix by vortexing until the NADP has dissolved. Place the tube in an ice bath. We have not found it necessary to filter-sterilize NADP solutions prepared this way but it can be done, if necessary, using a  $0.22\text{-}\mu\text{m}$  filter. Replace the left-over solution in the storage jar and return to the freezer for future use. Solutions of NADP stored in the freezer are stable for at least 6 months.

\* This amount of NADP applies to a formula weight of 765.4. Check the corrected formula weight indicated for each lot of NADP.

### 1 M glucose-6-phosphate

Use: S9 mix for mutagenicity assay

<i>Ingredient</i>	<i>Per 10 ml</i>
Glucose-6-phosphate (G-6-P)	2.82 g
Sterile distilled H <sub>2</sub> O	10 ml

Pre-weighed aliquots of glucose-6-phosphate are prepared as described for NADP and stored in desiccated jars in the freezer. Solutions of G-6-P can also be stored in the freezer and are stable for at least 6 months. If necessary, solutions may be filter-sterilized using a  $0.22\text{-}\mu\text{m}$  filter.

### S9 mix (rat liver microsomal enzymes + cofactors) \*

Use: Mutagenicity assay

#### *Per 50 ml*

<i>Ingredient</i>	<i>Standard S9 mix</i>	<i>High S9 mix</i>
Rat liver S9 (Aroclor-1254-induced)	2.0 ml (4%)	5.0 ml (10%)
MgCl <sub>2</sub> -KCl salts	1.0 ml	1.0 ml
1 M glucose-6-phosphate	0.25 ml	0.25 ml
0.1 M NADP	2.0 ml	2.0 ml
0.2 M phosphate buffer, pH 7.4	25.0 ml	25.0 ml
Sterile distilled H <sub>2</sub> O	19.75 ml	16.75 ml

\* Liver from other mammalian species such as hamster or mouse may be used. Other tissues may be used. The ingredients should be added in the reverse order indicated above so that the liver will be added to a buffered solution. The solution must be prepared fresh and kept on ice. All ingredients should be chilled. Any left over S9 or S9 mix should be discarded. *Never refreeze S9.*

## Ampicillin solution (8 mg/ml)

Use: Tests of ampicillin resistance

Master plates for R-factor strains

<i>Ingredient</i>	<i>Per liter</i>
Ampicillin trihydrate	0.8 g
Sodium hydroxide (0.02 N)	100 ml

We have never found it necessary to sterilize ampicillin solutions but they can be filtered through a 0.22- $\mu$ m membrane filter. Store in glass bottle at 4°C.

## Crystal violet solution (0.1%)

Use: Tests for crystal violet sensitivity (to confirm *rfa* mutation)

<i>Ingredient</i>	<i>Per liter</i>
Crystal violet	0.1 g
Distilled H <sub>2</sub> O	100 ml

Store at 4° in glass bottle with screw cap. Wrap the bottle with metal foil to protect against light.

## Minimal glucose plates

Use: Mutagenicity assay

<i>Ingredient</i>	<i>Per liter</i>
Agar	15 g
Distilled H <sub>2</sub> O	930 ml
50X VB salts	20 ml
40% glucose	50 ml

Add 15 g of agar to 930 ml of distilled H<sub>2</sub>O in a 2-liter flask. Autoclave for 20 min using slow exhaust. When the solution has cooled slightly, add 20 ml of sterile 50X VB salts and 50 ml of sterile 40% glucose. For mixing, a large magnetic stir bar can be added to the flask before autoclaving. After all the ingredients have been added, the solution should be stirred thoroughly. Pour 30 ml into each petri plate.

*Note:* The 50X VB salts and 40% glucose should be autoclaved separately.

## Histidine/biotin plates

Use: Master plates for non R-factor strains

Tests for histidine requirement

<i>Ingredient</i>	<i>Per liter</i>
Agar	15 g
Distilled H <sub>2</sub> O	914 ml
50X VB salts	20 ml
40% glucose	50 ml
Sterile histidine · HCl · H <sub>2</sub> O (2 g per 400 ml H <sub>2</sub> O)	10 ml
Sterile 0.5 mM biotin	6 ml

Autoclave the agar and water. Add the sterile 40% glucose, 50X VB salts, and histidine to the hot agar solution. Allow the solution to cool slightly. Add the sterile biotin. Mix, and pour the plates.

*Note:* A magnetic stir bar may be added before autoclaving to facilitate mixing. Autoclave the 50X VB salts, 40% glucose, and histidine solution separately.

#### Ampicillin plates and ampicillin/tetracycline \* plates

Use: Master plates for strains carrying the plasmids pKM101 and pKM101 + pAQ1 \*\*

Tests for ampicillin/tetracycline resistance

<i>Ingredient</i>	<i>Per liter</i>	<i>Plate concentration</i>
Agar	15 g	1.5%
Distilled H <sub>2</sub> O	910 ml	—
50X VB salts	20 ml	1X
40% glucose	50 ml	2.0%
Sterile histidine · HCl · H <sub>2</sub> O (2 g per 400 ml H <sub>2</sub> O)	10 ml	260 μM
Sterile 0.5 mM biotin	6 ml	3 μM
Sterile ampicillin solution (8 mg/ml 0.02 N NaOH)	3.15 ml	25 μg/ml
<hr/>		
Sterile tetracycline solution * (8 mg/ml 0.02 N HCl)	0.25 ml	2 μg/ml

Autoclave agar and water for 20 min. Add sterile glucose, 50X VB salts, and histidine to the hot solution. Mix. Cool to approximately 50°. Add sterile biotin and ampicillin solutions aseptically (we have not found it necessary to sterilize antibiotic solutions but this can be done, if necessary, using a 0.2-μm filter). \* *Tetracycline is added only for use with TA102 which is tetracycline-resistant.* It is essential not to exceed or fall below this concentration. See Levin et al. (1982b).

The 50X VB salts and 40% glucose solutions are sterilized separately by autoclaving for 20 min. Histidine and biotin solutions can be autoclaved or filter-sterilized.

Plates to be used for tests of tetracycline and/or ampicillin resistance can be stored for approx. 2 months at 4°C. After 2 months they should be tested for ampicillin/tetracycline activity with a non R-factor strain such as TA1535. Plates should be discarded if the non R-factor strain grows.

Master plates should be prepared within a few days after the agar is poured.

\*\* TA102 master plates should be discarded after 2 weeks. See *Master plates* section.

**Nutrient agar plates**

Use: 1. Tests for genotypes

(a) crystal violet sensitivity (*rfa*)(b) UV sensitivity ( $\Delta uvrB$ )

2. Tests for viability of bacteria

<i>Ingredient</i>	<i>Per liter</i>
Difco bacto nutrient broth *	8 g
NaCl	5 g
Agar	15 g
Distilled H <sub>2</sub> O	1 000 ml

Add the ingredients to a 2-liter flask containing a magnetic stir bar. Autoclave for 30 min. slow exhaust. Mix, and pour the plates.

\* Oxoid nutrient broth No. 2 can be substituted for Difco bacto nutrient broth, in which case 25 g are used and the NaCl is omitted. Since these rich agar plates are used when total growth of bacteria is required and not for mutagenicity assays, it makes no difference what kind of nutrient broth is used. Nutrient broth should not be used for master plates.

**LB agar plates**

Use: Growing the strains for lyophilization

<i>Ingredient</i>	<i>Per liter</i>
Bacto tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g
Agar	15 g
Distilled H <sub>2</sub> O	1 000 ml

Dissolve ingredients in water. Adjust to pH 7.0 with 1 N NaOH (approximately 2 ml/l). Add ingredients to a 2-liter flask containing a magnetic stir bar. Autoclave 30 min at 121°, slow exhaust. Mix, and pour the plates.

**References**

- Aeschbacher, H.U., and E. Ruch (1982) Urine-mediated Ames test: interactions. *Mutation Res.* 103, 127-131.
- Alvares, A.P., D.R. Bickers and A. Kappas (1973) Polychlorinated biphenyls: a new type of inducer of cytochrome P-448 in the liver, *Proc. Natl. Acad. Sci. (U.S.A.)*, 70, 1321-1325.
- Ames, B.N. (1971) The detection of chemical mutagens with enteric bacteria, in: A. Hollaender (Ed.), *Chemical Mutagens, Principles and Methods for Their Detection*. Plenum, New York. Vol. 1. pp. 267-282.

- Ames, B.N. (1972) A bacterial system for detecting mutagens and carcinogens, in: H.E. Sutton and M.I. Harris (Eds.), *Mutagenic Effects of Environmental Contaminants*, Academic Press, New York, pp. 57-66.
- Ames, B.N., and L. Haroun (1980) An overview of the Salmonella Mutagenicity Test, in: M.J. Coon, A.H. Conney, R.W. Estabrook, H.V. Gelboin, J.R. Gillette and P.J. O'Brien (Eds.), *Microsomes, Drug Oxidations, and Chemical Carcinogenesis*, Vol. II, Academic Press, New York, pp. 1025-1040.
- Ames, B.N., and J. McCann (1981) Validation of the Salmonella test: A reply to Rinkus and Legator, *Cancer Res.*, 41, 4192-4196.
- Ames, B.N., F.D. Lee and W.E. Durston (1973a) An improved bacterial test system for the detection and classification of mutagens and carcinogens, *Proc. Natl. Acad. Sci. (U.S.A.)*, 70, 782-786.
- Ames, B.N., W.E. Durston, E. Yamasaki and F.D. Lee (1973b) 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.
- Ames, B.N., H.O. Kammen and E. Yamasaki (1975a) Hair dyes are mutagenic: Identification of a variety of mutagenic ingredients, *Proc. Natl. Acad. Sci. (U.S.A.)*, 72, 2423-2427.
- Ames, B.N., J. McCann and E. Yamasaki (1975b) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test, *Mutation Res.*, 31, 347-364.
- Arimoto, S., K. Negishi and H. Hayatsu (1981) A modification of the Ames test procedure: Accelerated growth of the His<sup>+</sup> revertants, *Mutation Res.*, 91, 407-411.
- Ashwood-Smith, M.J. (1977) Stability of microsomal enzymes associated with the conversion of carcinogens to bacterial mutagens (Ames' Salmonella/microsome test) to freezing and thawing, *Cryobiology*, 14, 240-244.
- Ashwood-Smith, M.J. (1980) Stability of frozen microsome preparations for use in the Ames' Salmonella mutagenicity assay, *Mutation Res.*, 69, 199-200.
- Barnes, W., and R.N. Husson (1982) personal communication.
- Barnes, W., E. Tuley and E. Eisenstadt (1982) Base-sequence analysis of His<sup>+</sup> revertants of the *hisG46* missense mutation in *Salmonella typhimurium*, *Environ. Mutagen.*, 4, 297 (abstr. Aa-1).
- Bartsch, H., C. Malaveille and R. Montesano (1975a) Human, rat and mouse liver-mediated mutagenicity of vinyl chloride in *S. typhimurium* strains, *Int. J. Cancer*, 15, 429-437.
- Bartsch, H., C. Malaveille, R. Montesano and L. Tomatis (1975b) Tissue-mediated mutagenicity of vinylidene chloride and 2-chlorobutadiene in *Salmonella typhimurium*, *Nature (London)*, 255, 641-643.
- Bartsch, H., C. Malaveille and R. Montesano (1975c) In vitro metabolism and microsome-mediated mutagenicity of dialkylnitrosamines in rat, hamster, and mouse tissues, *Cancer Res.*, 35, 644-651.
- Bartsch, H., C. Malaveille, A.-M. Camus, G. Martel-Planche, G. Brun, A. Hautefeuille, N. Sabadie, A. Barbin, T. Kuroki, C. Drevon, C. Piccoli and R. Montesano (1980) Validation and comparative studies on 180 chemicals with *S. typhimurium* strains and V79 Chinese hamster cells in the presence of various metabolizing systems, *Mutation Res.*, 76, 1-50.
- Belser, W.L., Jr., S.D. Shaffer, R.D. Bliss, P.M. Hynds, L. Yamamoto, J.N. Pitts, Jr. and J.A. Winer (1981) A standardized procedure for quantification of the Ames Salmonella/mammalian-microsome mutagenicity test, *Environ. Mutagen.*, 3, 123-139.
- Bernstein, L., J. Kaldor, J. McCann and M.C. Pike (1982) An empirical approach to the statistical analysis of mutagenesis data from the Salmonella test, *Mutation Res.*, 97, 267-281.
- Bridges, B.A. (1978) On the detection of volatile liquid mutagens with bacteria: Experiments with dichlorvos and epichlorhydrin, *Mutation Res.*, 54, 367-371.
- Brown, J.P., and P.S. Dietrich (1979) Mutagenicity of plant flavonols in the Salmonella/mammalian microsome test: Activation of flavonol glycosides by mixed glycosidases from rat cecal bacteria and other sources, *Mutation Res.*, 66, 223-240.
- Commoner, B., A.J. Vithayathil and J.I. Henry (1974) Detection of metabolic carcinogen intermediates in urine of carcinogen-fed rats by means of bacterial mutagenesis, *Nature (London)*, 249, 850-852.
- Czygan, P., H. Greim, A.J. Garro, F. Hutterer, F. Schaffner, H. Popper, O. Rosenthal and D.Y. Cooper (1973) Microsomal metabolism of dimethylnitrosamine and the cytochrome P-450 dependency of its activation to a mutagen, *Cancer Res.*, 33, 2983-2986.
- De Serres, F.J., and M.D. Shelby (1979) Recommendations on data production and analysis using the Salmonella/microsome mutagenicity assay, *Mutation Res.*, 64, 159-165.

- Durston, W.E., and B.N. Ames (1974) A simple method for the detection of mutagens in urine: studies with the carcinogen 2-acetylaminofluorene, *Proc. Natl. Acad. Sci. (U.S.A.)*, 71, 737-741.
- Ecobichon, D.J., and A.M. Comeau (1974) Comparative effects of commercial Aroclors on rat liver enzyme activities, *Chem.-Biol. Interact.*, 9, 341-350.
- Friederich, U., H.U. Aeschbacher, J.P. Seiler and F.E. Wurgler (1982) The Salmonella/microsome assay: some possible causes for interlaboratory variations, *Mutation Res.*, 103, 133-140.
- Garner, R.C., E.C. Miller and J.A. Miller (1972) Liver microsomal metabolism of aflatoxin B<sub>1</sub> to a reactive derivative toxic to *Salmonella typhimurium* TA1530, *Cancer Res.*, 32, 2058-2066.
- Gingell, R., and R. Walker (1971) Mechanisms of azo reduction by *Streptococcus faecalis*, II. The role of soluble flavins, *Xenobiotica*, 1, 231-239.
- Green, M.H.L., and W.J. Muriel (1976) Mutagen testing using Trp<sup>+</sup> reversion in *Escherichia coli*, *Mutation Res.*, 38, 3-32.
- Herbold, B.A. (1982) Preliminary data from an international survey on the sensitivity of Ames tester strains, *Mutation Res.*, 97, 191-192.
- Hollstein, M., J. McCann, F. Angelosanto and W. Nichols (1979) Short-term tests for carcinogens and mutagens, *Mutation Res.*, 65, 133-226.
- Isono, K., and J. Yourno (1974) Chemical carcinogens as frameshift mutagens: Salmonella DNA sequence sensitive to mutagenesis by polycyclic carcinogens, *Proc. Natl. Acad. Sci. (U.S.A.)*, 71, 1612-1617.
- Jose, J. (1979) Photomutagenesis by chlorinated phenothiazine tranquilizers, *Proc. Natl. Acad. Sci. (U.S.A.)*, 76, 469-472.
- Kado, N.Y., D. Langley and E. Eisenstadt (1982) personal communication.
- Katz, A.J. (1979) Design and analysis of experiments on mutagenicity. II. Assays involving microorganisms, *Mutation Res.*, 64, 61-77.
- Kier, L.D., E. Yamasaki and B.N. Ames (1974) Detection of mutagenic activity in cigarette smoke condensates, *Proc. Natl. Acad. Sci. (U.S.A.)*, 71, 4159-4163.
- Langer, P.J., W.G. Shanabruch and G.C. Walker (1981) Functional organization of plasmid pKM101. *J. Bacteriol.*, 145, 1310-1316.
- LaPage, S.P., J.E. Shelton, T.G. Mitchell and A.R. MacKenzie (1970) in J.R. Norris and D.W. Ribbons (Eds.), *Methods in Microbiology: Culture Collections and Preservation of Bacteria*, Chapter II, Academic Press, New York, pp. 135-228.
- Levin, D.E., E. Yamasaki and B.N. Ames (1982a) A new Salmonella tester strain for the detection of frameshift mutagens: A run of cytosines as a mutational hot-spot, *Mutation Res.*, 94, 315-330.
- Levin, D.E., M.C. Hollstein, M.F. Christman, E.A. Schwiens and B.N. Ames (1982b) A new Salmonella tester strain (TA102) with A:T base pairs at the site of mutation detects oxidative mutagens, *Proc. Natl. Acad. Sci. (U.S.A.)*, 79, 7445-7449.
- Litterst, C.L., and E.J. van Loon (1974) Time-course of induction of microsomal enzymes following treatment with polychlorinated biphenyl, *Bull. Environ. Contam. Toxicol.*, 11, 206-212.
- Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) Protein measurement with Folin phenol reagent, *J. Biol. Chem.*, 193, 265-275.
- Margolin, B.H., N. Kaplan and E. Zeiger (1981) Statistical analysis of the Ames Salmonella/microsome test, *Proc. Natl. Acad. Sci. (U.S.A.)*, 78, 3779-3783.
- Maron, D., J. Katzenellenbogen and B.N. Ames (1981) Compatibility of organic solvents with the Salmonella/microsome test, *Mutation Res.*, 88, 343-350.
- Matsushima, T., M. Sawamura, K. Hara and T. Sugimura (1976) A safe substitute for polychlorinated biphenyls as an inducer of metabolic activation system, in: F.J. de Serres, J.R. Fouts, J.R. Bend and R.M. Philpot (Eds.), *In Vitro Metabolic Activation in Mutagenesis Testing*, Elsevier/North-Holland, Amsterdam, pp. 85-88.
- Matsushima, T., H. Matsumoto, A. Shirai, M. Swamura and T. Sugimura (1979) Mutagenicity of the naturally occurring carcinogen cycasin and synthetic methylazoxymethanol conjugates in *Salmonella typhimurium*, *Cancer Res.*, 39, 3780-3782.
- Matsushima, T., T. Sugimura, M. Nagao, T. Yahagi, A. Shirai and M. Sawamura (1980) Factors modulating mutagenicity in microbial tests, in: K.H. Nörpöth and R.C. Garner (Eds.), *Short-Term Test Systems for Detecting Carcinogens*, Springer, Berlin, pp. 273-285.

- McCann, J., and B.N. Ames (1976) Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals: Discussion, Proc. Natl. Acad. Sci. (U.S.A.), 73, 950-954.
- McCann, J., and B.N. Ames (1977) The Salmonella/microsome mutagenicity test: Predictive value for animal carcinogenicity, in: H.H. Hiatt, J.D. Watson and J.A. Winsten (Eds.), Origins of Human Cancer, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 1431-1450.
- McCann, J., E. Choi, E. Yamasaki and B.N. Ames (1975a) Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals, Proc. Natl. Acad. Sci. (U.S.A.), 72, 5135-5139.
- McCann, J., N.E. Springarn, J. Kobori and B.N. Ames (1975b) Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids, Proc. Natl. Acad. Sci. (U.S.A.), 72, 979-983.
- McCoy, E.C., H.S. Rosenkranz and R. Mermelstein (1981) Evidence for the existence of a family of bacterial nitroreductases capable of activating nitrated polycyclics to mutagens, Environ. Mutagen., 3, 421-427.
- Mortelmans, K. (1982) Personal communication.
- Nagao, M., T. Yahagi, Y. Seino, T. Sugimura and N. Ito (1977) Mutagenicities of quinoline and its derivatives, Mutation Res., 42, 335-342.
- NCI (1978a) Bioassay of 2,4-diaminoanisole sulfate for possible carcinogenicity, NCI Carcinogenesis Technical Report Series No. 84, DHEW publication No. (NIH) 78-1334.
- NCI (1978b) Bioassay of tris(2,3-dibromopropyl)phosphate for possible carcinogenicity, NCI Carcinogenesis Technical Report Series No. 76, DHEW publication No. (NIH) 78-1326.
- NCI (1979a) Bioassay of 2,4-diaminotoluene for possible carcinogenicity, NCI Carcinogenesis Technical Report Series No. 162, DHEW publication No. (NIH) 79-1718.
- NCI (1979b) Bioassay of 2-nitro-*o*-phenylenediamine for possible carcinogenicity, NCI Carcinogenesis Technical Report Series No. 169, DHEW publication No. (NIH) 79-1725.
- Ohgake, H., N. Matsukura, K. Morino, T. Kawachi, T. Sugimura, K. Morita, H. Tokiwa and T. Hirota (1982) Carcinogenicity in rats of the mutagenic compounds 1-nitropyrene and 3-nitrofluoranthene, Cancer Lett., 15, 1-7.
- Ong, T.-M., Mukhtar, C.R. Wolf and E. Zeiger (1980) Differential effects of cytochrome P450-inducers on promutagen activation capabilities and enzymatic activities of S9 from rat liver, J. Environ. Pathol. Toxicol., 4, 55-65.
- Prival, M.J., and V.D. Mitchell (1981) Influence of microsomal and cytosolic fractions from rat, mouse, and hamster liver on the mutagenicity of dimethylnitrosamine in the Salmonella plate incorporation assay, Cancer Res., 41, 4361-4367.
- Prival, M.J., and V.D. Mitchell (1982) Analysis of a method for testing azo dyes for mutagenic activity in *Salmonella typhimurium* in the presence of flavin mononucleotide and hamster liver S9, Mutation Res., 97, 103-116.
- Prival, M.J., V.D. King and A.T. Sheldon, Jr. (1979) The mutagenicity of dialkyl nitrosamines in the Salmonella plate assay, Environ. Mutagen., 1, 95-104.
- Purchase, I.F.H., E. Longstaff, J. Ashby, J.A. Styles, D. Anderson, P.A. Lefevre and F.R. Westwood (1976) Evaluation of six short term tests for detecting organic chemical carcinogens and recommendations for their use, Nature (London), 264, 624-627.
- Raineri, R., J.A. Poiley, A.W. Andrews, R.J. Pienta and W. Lijinsky (1981) Greater effectiveness of hepatocyte and liver S9 preparations from hamsters than rat preparations in activating *N*-nitroso compounds to metabolites mutagenic to Salmonella, J. Natl. Cancer Inst., 67, 1117-1122.
- Rannug, U., A. Johansson, C. Ramel and C.A. Wachmeister (1974) The mutagenicity of vinyl chloride after metabolic activation, Ambio, 3, 194-197.
- Rinkus, S.J., and M.S. Legator (1979) Chemical characterization of 465 known or suspected carcinogens and their correlation with mutagenic activity in the *Salmonella typhimurium* system, Cancer Res., 39, 3289-3318.
- Rinkus, S.J., and M.S. Legator (1981) Salmonella revisited: A reply to Ames and McCann, Cancer Res., 41, 4196-4203.
- Rosenkranz, H.S., and W.T. Speck (1975) Mutagenicity of metronidazole: Activation by mammalian liver microsomes, Biochem. Biophys. Res. Commun., 66, 520-525.
- Rosenkranz, H.S., and W.T. Speck (1976) Activation of nitrofurantoin to a mutagen by rat liver nitroreductase, Biochem. Pharmacol., 25, 1555-1556.

- Rosenkranz, H.S., E.C. McCoy, D.R. Sanders, M. Butler, D.K. Kiriazides and R. Mermelstein (1980a) Nitropyrenes: Isolation, identification and reduction of mutagenic impurities in carbon black and toners, *Science*, 209, 1039-1043.
- Rosenkranz, H.S., G. Karpinsky and E.C. McCoy (1980b) Microbial assays: Evaluation and application to the elucidation of the etiology of colon cancer, in: K. Norpoth and R.C. Garner (Eds.), *Short-Term Test Systems for Determining Carcinogens*, Springer, Berlin, pp. 19-57.
- Rosenkranz, H.S., E.C. McCoy, R. Mermelstein and W.T. Speck (1981) A cautionary note on the use of nitroreductase-deficient strains of *Salmonella typhimurium* for the detection of nitroarenes as mutagens in complex mixtures including diesel exhausts, *Mutation Res.*, 91, 103-105.
- Schmoltdt, A., H.F. Bente and R. Frühling (1974) Induction of rat liver enzymes by polychlorinated biphenyls (PCBs) in dependence on the dose and chlorine content, *Arch. Toxicol.*, 32 (1974) 69-81.
- Schuetzle, D., T.J. Prater, T. Riley, A. Durisin and I. Salmee (1980) Analysis of nitrated derivatives of PAH and determination of their contribution to Ames assay mutagenicity for diesel particulate extracts, 5th Intern. Symp. Polynuclear Aromatic Hydrocarbons, Columbus, OH, abstracts.
- Schuetzle, D., F.S.-C. Lee, T.J. Prater and S.B. Tejada (1981) The identification of polynuclear aromatic hydrocarbon (PAH) derivatives in mutagenic fractions of diesel particulate extracts, *Int. J. Environ. Anal. Chem.*, 9, 93-144.
- Shahin, M.M., P. Andrillon, N. Goetz, P. Bore, A. Bugaut and G. Kalopissis (1979) Studies on the mutagenicity of *p*-phenylenediamine in *Salmonella typhimurium*, Presence of PCBs in rat-liver microsomal fraction induced by Aroclor, *Mutation Res.*, 68, 327-336.
- Shanabruch, W.G., and G.C. Walker (1980) Localization of the plasmid (pKM101) gene(s) involved in *recA*<sup>+</sup> *lexA*<sup>-</sup> dependent mutagenesis, *Mol. Gen. Genet.*, 179, 289-297.
- Simmon, V.F. (1981) Applications of the Salmonella/microsome assay, in: H.F. Stich and R.H.C. San (Eds.), *Short-Term Tests for Chemical Carcinogens*, Springer, New York, pp. 120-126.
- Simmon, V.F., K. Kauhanen and R.G. Tardiff (1977) Mutagenic activities of chemicals identified in drinking water, in: D. Scott, B.A. Bridges and F.H. Sobels (Eds.), *Progress in Genetic Toxicology*, Elsevier/North-Holland, Amsterdam, pp. 249-258.
- Stead, A.G., V. Hasselblad, J.P. Creason and L. Claxton (1981) Modeling the Ames test, *Mutation Res.*, 85, 13-27.
- Sugimura, T. (1982) A view of a cancer researcher on environmental mutagens, in: H. Takebe (Ed.), *Proceedings of the Third International Conference on Environmental Mutagens*, University of Tokyo Press/Alan R. Liss, pp. 3-20.
- Sugimura, T., S. Sato, M. Nagao, T. Yahagi, T. Matsushima, Y. Seino, M. Takeuchi and T. Kawachi (1976) Overlapping of carcinogens and mutagens, in: P.N. Magee et al. (Eds.), *Fundamentals in Cancer Prevention*, University of Tokyo Press, Tokyo, pp. 191-215.
- Sugimura, T., M. Nagao, T. Kawachi, M. Honda, T. Yahagi, Y. Seino, S. Sato, N. Matsukura, T. Matsushima, A. Shirai, M. Sawamura and H. Matsumoto (1977) Mutagen-carcinogens in food, with special reference to highly mutagenic pyrolytic products in broiled foods, in: H.H. Hiatt, J.D. Watson and J.A. Winsten (Eds.), *Origins of Human Cancer: Cold Spring Harbor Laboratory*, Cold Spring Harbor, NY, pp. 1561-1577.
- Tamura, G., C. Gold, A. Ferro-Luzzi and B.N. Ames (1980) Fecalase: A model for activation of dietary glycosides to mutagens by intestinal flora, *Proc. Natl. Acad. Sci. (U.S.A.)*, 77, 4961-4965.
- Venitt, S., and C. Crofton-Sleigh (1979) Bacterial mutagenicity tests of phenazine methosulfate and three tetrazolium salts, *Mutation Res.*, 68, 107-116.
- Vithayathil, A.J., B. Commoner, S. Nair and P. Madyastha (1978) Isolation of mutagens from bacterial nutrients containing beef extract, *J. Toxicol. Environ. Health*, 4, 189-202.
- Vogel, H.J., and D.M. Bonner (1956) Acetylornithinase of *Escherichia coli*: partial purification and some properties, *J. Biol. Chem.*, 218, 97-106.
- Waleh, N.S., S.J. Rapport and K. Mortelmans (1982) Development of a toxicity test to be coupled to the Ames Salmonella assay and the method of construction of the required strains, *Mutation Res.*, 97, 247-256.
- Walker, G.C., and P.P. Dobson (1979) Mutagenesis and repair deficiencies of *Escherichia coli umuC* mutants are suppressed by the plasmid pKM101, *Mol. Gen. Genet.*, 172, 17-24.

- Watabe, T., T. Ishizuka, M. Isobe and N. Ozawa (1982) A 7-hydroxymethyl sulfate ester as an active metabolite of 7,12-dimethylbenz[ $\alpha$ ]anthracene, *Science*, 215, 403-404.
- Weeks, R.W., Jr., and M.J. McLeod (1980) Permeation of protective garment material by liquid halogenated ethanes and a polychlorinated biphenyl, Los Alamos National Laboratory Report No. LA-8572-MS.
- Weeks, R.W., Jr., and M.J. McLeod (1982) Permeation of protective garment material by liquid benzene and by tritiated water, *Am. Indust. Hyg. Assoc. J.*, 43, 201-211.
- Weinstein, D., and T.M. Lewinson (1978) A statistical treatment of the Ames mutagenicity assay, *Mutation Res.*, 51, 433-434.
- Weisburger, E., A.B. Russfield, F. Homburger, J.H. Weisburger, E. Boger, C.G. Van Dongen and K.C. Chu (1978) Testing of twenty-one environmental aromatic amines or derivatives for long-term toxicity or carcinogenicity, *J. Environ. Pathol. Toxicol.*, 2, 325-356.
- Xu, X.B., J.P. Nachtman, Z.L. Jin, E.T. Wei, S.M. Rappaport and A.L. Burlingame (1982) Isolation and identification of mutagenic nitro-PAH in diesel-exhaust particulates, *Anal. Chim. Acta*, 136, 163-174.
- Yahagi, T., M. Degawa, Y. Seino, T. Matsushima, M. Nagao, T. Sugimura and Y. Hashimoto (1975) Mutagenicity of carcinogenic azo dyes and their derivatives, *Cancer Lett.*, 1, 91-96.
- Yahagi, T., M. Nagao, Y. Seino, T. Matsushima, T. Sugimura and M. Okada (1977) Mutagenicities of *N*-nitrosamines on *Salmonella*, *Mutation Res.*, 48, 121-130.
- Yamanaka, H., M. Nagao, T. Sugimura, T. Furuya, A. Shirai and T. Matsushima (1979) Mutagenicity of pyrrolizidine alkaloids in the *Salmonella*/mammalian-microsome test, *Mutation Res.*, 68, 211-216.
- Yamasaki, E. and B.N. Ames (1977) Concentration of mutagens from urine by adsorption with the nonpolar resin XAD-2: Cigarette smokers have mutagenic urine, *Proc. Natl. Acad. Sci. (U.S.A.)*, 74, 3555-3559.
- Zeiger, E. (1982) Personal communication.
- Zeiger, E., and D.A. Pagano (1982) Comparative mutagenicity of dimethylaminoazobenzene and analogues in *Salmonella*, *Carcinogenesis*, 3, 559-561.
- Zeiger, E., R.S. Chhabra and B.H. Margolin (1979) Effects of the hepatic S9 fraction from Aroclor-1254-treated rats on the mutagenicity of benzo[*a*]pyrene and 2-aminoanthracene in the *Salmonella*/microsome assay, *Mutation Res.*, 64, 379-389.
- Zeiger, E., D.A. Pagano and I.G.C. Robertson (1981) A rapid and simple scheme for confirmation of *Salmonella* tester strain phenotype, *Environ. Mutagen.*, 3, 205-209.