

CHAPTER 9

The Detection of Chemical Mutagens with Enteric Bacteria*

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with an addendum by

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I. INTRODUCTION

Mutagens alter DNA. As the DNA of all organisms has the same double helical structure and the same four nucleotides, any organism may be used as an indicator system for mutagens. Bacteria have numerous advantages for the detection of mutagens, and we discuss these and describe various methods and strains. We also discuss the validity of the bacterial tests and their pertinence to human mutagenesis and carcinogenesis.

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II. DISCUSSION

A. Advantages of Using Bacterial Test Systems for the Detection of Mutagens

Any test system for mutagens should be calibrated against the known mutagens to determine the ease and sensitivity of the test in detecting these compounds before trying new substances. We believe bacteria are the system of choice for mass screening of new compounds on the basis of simplicity, sensitivity, economy, and range of compounds detected.

1. *The Simplicity of the Plate Test*

The advantages of using bacteria for testing mutagens have been discussed by a number of authors and various methods have been examined and used for testing a variety of compounds (Demerec *et al.*, 1951; Szybalski, 1958). We have found that the method of adding the mutagen directly to a lawn of bacteria on a petri plate (Iyer and Szybalski, 1958) to be the most convenient. In this paper we discuss the use of special bacterial strains that make this method capable of detecting many more types of mutagens and much more sensitive and diagnostic of the different classes of mutagens.

Most of the tests have been designed to be done on petri plates. A small sample (about 1 mg) of the suspected mutagen is placed in the center of a petri plate that has been seeded with a lawn of bacteria that cannot grow because of a mutation. If the mutagen can cause the particular mutation to revert in an occasional bacterium, it will enable that bacterium to grow and form a colony. Therefore, a circle of colonies will appear around the spot of mutagen after about a day and a half of incubation. The mutagen forms a concentration gradient as it diffuses from the point of application, and one often sees a clear central area, where the high concentration of the mutagen has inhibited all the bacteria, surrounded by a zone of mutant colonies. The diffusion of the mutagen in the agar allows one, in effect, to test a wide range of concentrations on a single plate (Fig. 1). Because all the revertants caused by the mutagen appear as a ring around the spot of application, occasional contaminants or some spontaneous mutants randomly distributed around the plate do not interfere with the test, and the interpretation is usually unambiguous.

Using this test system a person with minimal training can test several hundred compounds every day. No elaborate equipment is necessary and the cost of supplies is small.

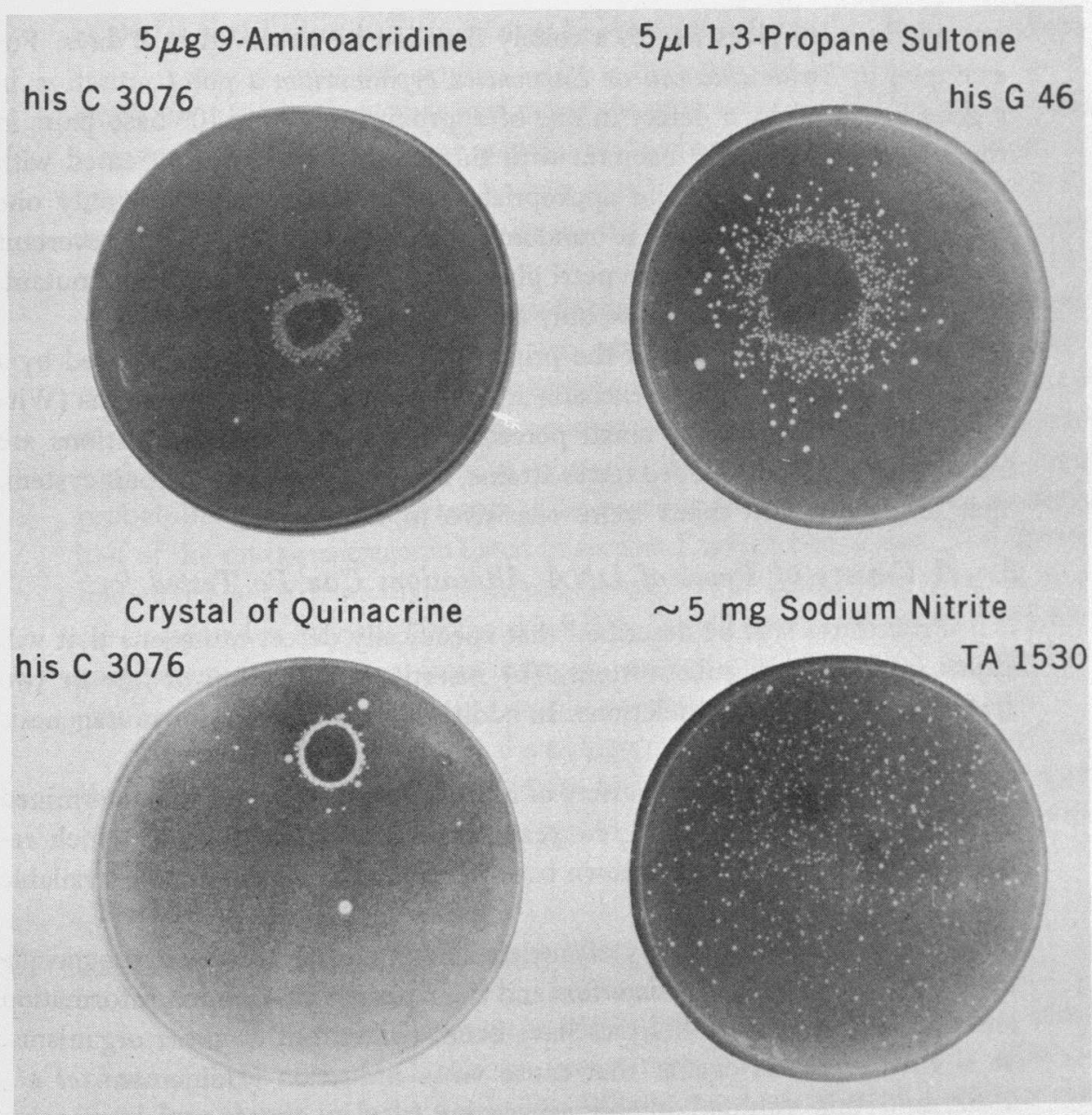


FIGURE 1. Photographs of 90-mm petri plates showing the reversion of tester strains by various mutagens added at a spot on the plate. Each bacterial colony appears as a white spot. Where the mutagen inhibits, the ring of mutants appears outside of the zone of inhibition.

2. Sensitivity: Strains Lacking Repair

A compound that alters only one nucleotide base at random out of a million is a powerful mutagen that can cause a high proportion of lethal events or defective children in a population. This is because every nucleotide is important in the DNA code. Thus one must use large populations in testing for mutagens, as mutation is a rare event for any particular nucleotide or gene. In the bacterial test system even rare mutational events may be detected readily, since about 5×10^8 bacteria are exposed to the mutagen on a petri plate. Even if only a few bacteria are mutated at the appropriate

nucleotide, each gives rise to a colony that can be observed in 2 days. For example, in *Escherichia coli* or *Salmonella typhimurium* a point mutation in a gene is caused by a defect in one of approximately 4×10^6 base pairs in the genome. If 5×10^8 bacteria with this point mutation are treated with a mutagen that induces the appropriate nucleotide alteration in only one nucleotide per bacterium at random, then approximately 125 revertant colonies would appear on the petri plate. Depending on the starting mutant, the spontaneous rate might be only a few percent of this.

In bacteria almost all of the primary damage to the DNA caused by a mutagen is repaired by the excision and recombination repair systems (Witkin, 1969) so that only a small percentage of the potential mutations are induced. We have developed tester strains, lacking the excision repair system, that are 10 to 100 times more sensitive to mutagens.

3. *A Variety of Types of DNA Alterations Can Be Tested for*

Procedures will be described that specifically detect mutagens that will cause (a) base-pair substitutions, (b) insertion or deletion of one or two base pairs, and (c) large deletions. In addition, a general test for mutagenesis will be described.

Protein sequences of a variety of mutant enzymes have been determined in enteric bacteria. Within a few years, a set of mutants each of which responds only to a particular known base-pair change should become available (see addendum).

Simple tests for a variety of nucleic acid alterations are becoming available in *E. coli* and *S. typhimurium* and their phages as so much information and so many genetic techniques have been worked out in these organisms. It is easy to detect agents that cause virus induction (Heinemann *et al.*, 1967, and this volume), phenotypic curing (that is, agents such as streptomycin that cause mistakes in the genetic coding system during protein synthesis; Gorini and Kataja, 1965) (see section G), mistakes in transcribing DNA into RNA, episome curing (Hirota, 1960), insertions of genetic material, DNA replication inhibition, and agents such as caffeine that inhibit repair systems (Sideropoulos and Shankel, 1968).

B. **Validity of the Bacterial System as a Test for Mutagens and Carcinogens for Humans**

In normal drug testing it is absurd to extrapolate from bacteria to humans. In testing mutagens it is sensible because in both organisms the mutagen is reacting with DNA. This is borne out by the finding that, in general, mutagens for higher organisms are mutagens for microorganisms and *vice versa*, where extensive tests have been done. In some cases in humans a mutagen effective on bacteria may be detoxified, or never reach the DNA

of the germ line, or mutations may be repaired effectively; and thus a bacterial mutagen may not appear to be a mutagen for humans. It is worth emphasizing, however, that one molecule of a mutagen is enough to cause a mutation and that if a large population is exposed to a "weak" mutagen it may still be a hazard to the human germ line. Since no repair system is completely effective, there may be no such thing as a completely safe dose of a mutagen.

In addition, a high proportion of the known mutagens are carcinogenic and a high proportion of the known proximal carcinogens are mutagenic. Although the correlation is not absolute and although the theoretical basis is not clear (perhaps only agents causing one type of mutation are carcinogenic), what is clear is that if a substance mutates bacteria there is a high probability that it will turn out to be carcinogenic for humans (more than half of the mutagenic agents listed in section F are carcinogenic). The simple bacterial assay for mutagens should be useful for identifying possible new carcinogens for humans (Szybalski, 1958). It also may be useful in purifying carcinogenic substances from complex natural products such as cigarette smoke, if there is both mutagenic and carcinogenic activity.

My general feeling is that if a compound is a mutagen in any organism, then it should not be used on humans unless there is definitive evidence that it is neither mutagenic nor carcinogenic in animals, or unless the benefit outweighs the possible risk.

C. Validity of a Negative Result in the Bacterial Test System

A class of potential mutagens that is not detected with bacteria is composed of those compounds that are not mutagenic themselves but are metabolized by animals to form a mutagen. The natural compound cycasin is not a mutagen in the bacterial test (Smith, 1966). It is split by the intestinal flora of humans to glucose and the toxic methylazoxymethanol, which is a powerful mutagen, teratogen, and carcinogen (Spatz *et al.*, 1967). The methylazoxymethanol is a mutagen in the bacterial test (Smith, 1966). The host-mediated assay (see chapter in this book) might be useful for detecting compounds that are missed in a direct test if the sensitivity can be increased so that it can detect the known types of mutagens.

If a mutagen is most active at a pH other than pH 7, the pH of the bacterial growth medium in the agar plates, it is possible that it would not be detected on the petri plates. For example, a known mutagen such as nitrite is more active at low pH, where it is in the form of nitrous acid. Geneticists usually use nitrite at a pH about 4.5 when they are using it as a mutagen (e.g., Schwartz and Beckwith, 1969). Even at pH 7, however, strain TA1530 is reverted by nitrite (Fig. 1), though the less sensitive strain hisG46 is not. (The nitrite added as a color fixative to bacon, ham, frankfurters,

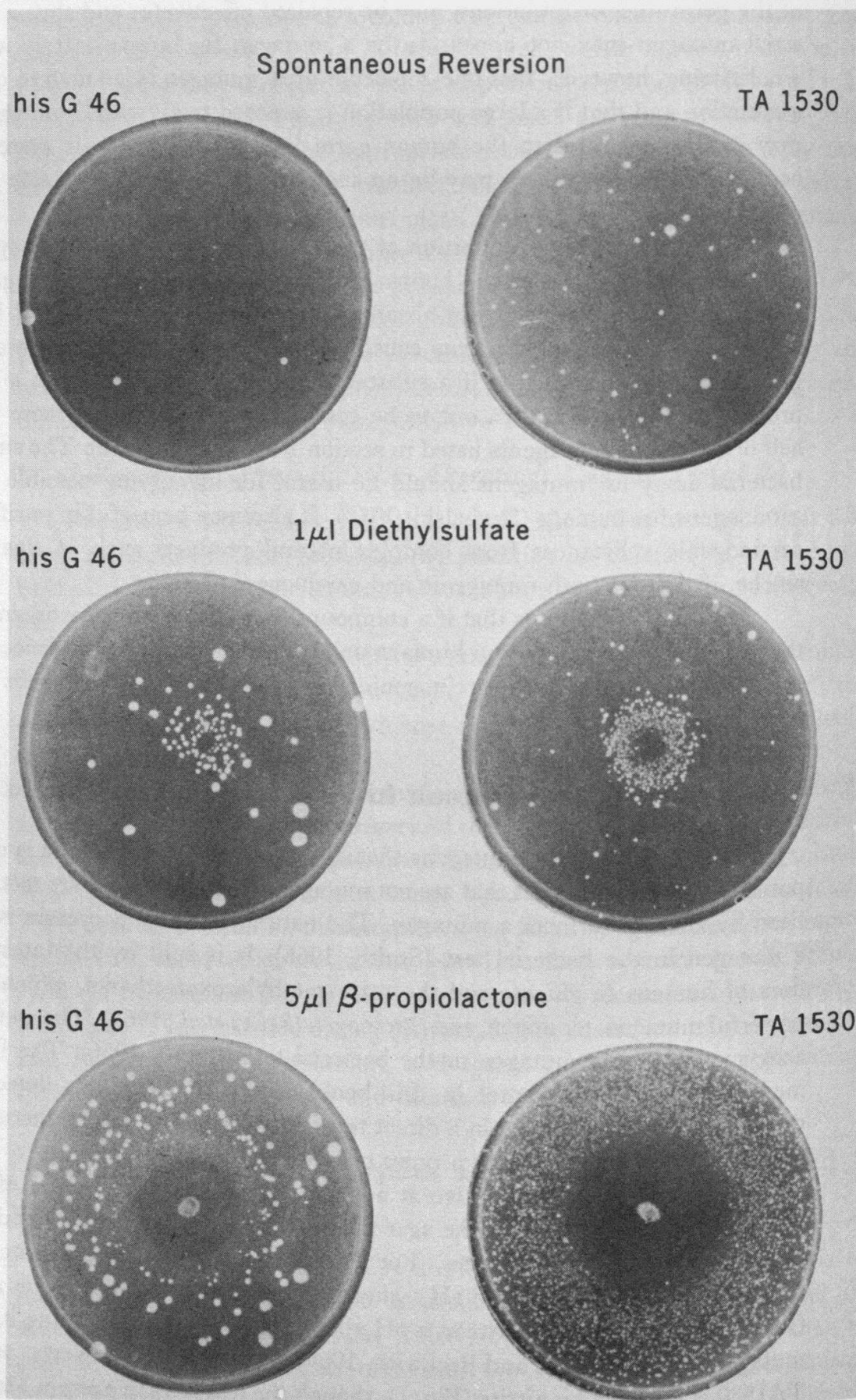
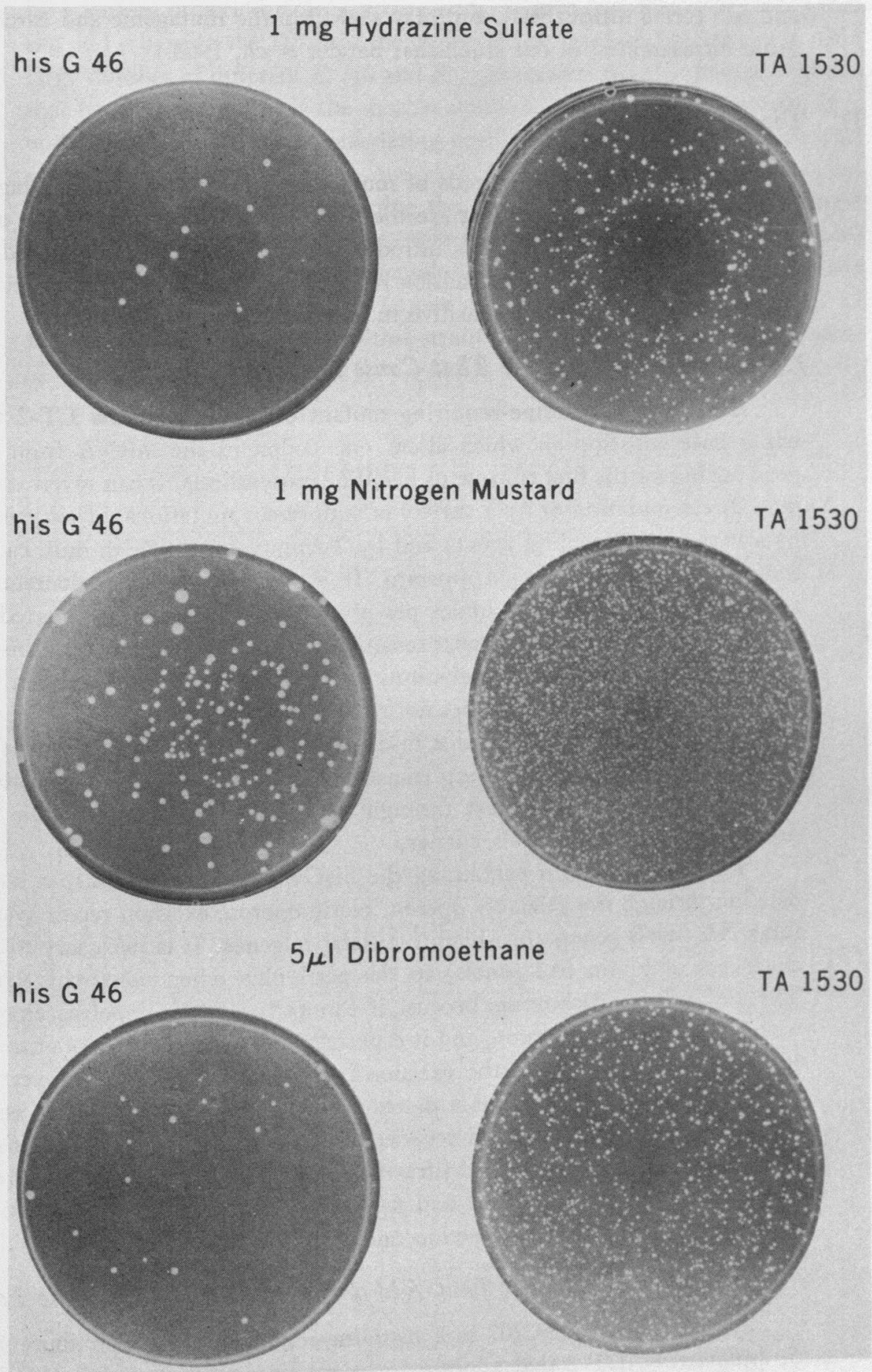


FIGURE 2. Photographs showing the reversion of mutation *hisG46* by various mutagens.



The double mutant *hisG46-uvrB* (TA1530) is compared to the *hisG46* strain.

and fish forms nitrous acid, and may also form the mutagenic and carcinogenic nitrosamines, in our stomachs; Sander *et al.*, 1968.)

D. Tester Strains

We have screened hundreds of mutants against a variety of mutagens and chosen three mutants that are the most sensitive to reversion by each class of mutagen. We have then introduced into these three mutants a deletion through the *uvrB* gene (excision repair); these strains then lack excision repair and are much more sensitive to reversion by mutagens.

1. Detection of Mutagens That Cause Base-Pair Substitutions

HisG46 is a histidine-requiring mutant of *S. typhimurium* LT-2 that has a base substitution which alters one codon in the mRNA from the gene coding for the first enzyme of histidine biosynthesis. It can revert either by a direct mutation or by a variety of suppressor mutations. It is reverted by a variety of alkylating agents and by 2-aminopurine (which only causes transitions) and by nitrogen mustard. It has a relatively low spontaneous reversion rate (about ten colonies per plate), is extremely well reverted by all the mutagens, and is the most sensitive to reversion of all the strains we have examined for base-pair substitutions. We have found no mutagen that reverts any other strain that does not revert G46, with the natural exception of the acridine-type mutagens that insert and delete bases. It is clear that the strain will detect agents causing transitions. Furthermore, since the strain can revert directly and revert through suppressors, I suspect that it will detect all possible base-pair changes.

TA1530 is a strain containing the *hisG46* mutation and also a single deletion through the galactose operon, biotin operon, excision repair system for DNA (*uvrB* gene), and chlorate-resistance genes. It is necessary to add an excess of biotin (0.1 μ mole) to the petri plate when using this strain. The strain is nonpathogenic because it cannot make its lipopolysaccharide without the galactose operon, and it is especially sensitive to a variety of mutagens because it is lacking the excision repair system. It cannot revert for *gal*, *uvrB*, or *bio* because it is a deletion. Compared to *hisG46*, it is about seven times more sensitive to reversion by diethylsulfate, about 30 times more sensitive to reversion by hydrazine, about 100 times more sensitive to reversion by nitrogen mustard and β -propiolactone, and has about a three times higher spontaneous reversion rate (Fig. 2, left and right).

2. Detection of Mutagens That Add or Delete One or Two Base Pairs

a. *HisC207*. *HisC207* is a histidine-requiring frameshift mutant of *S. typhimurium* LT-2 that appears to be missing one or two base pairs in the aminotransferase gene of the histidine operon. The mutant is reverted

by a variety of acridine (ICR compounds) (Ames and Whitfield, 1966) that can add a base to the DNA in the region of the original lost base pair. Of a large number of different *E. coli* and *S. typhimurium* frameshift-type mutants that have been tried, it is the most sensitive to these ICR compounds. It is not reverted by any of the alkylating agents or agents that cause base-pair substitutions.

TA1531 is a strain containing the *hisC207* mutation and also a single deletion through the galactose operon, biotin operon, *uvrB* gene, and chlorate-resistance gene (see discussion of TA1530). One microgram of the acridine half-mustard ICR191 (Ames and Whitfield, 1966) gave about 17 times as many revertants with this strain as with *hisC207*. The spontaneous

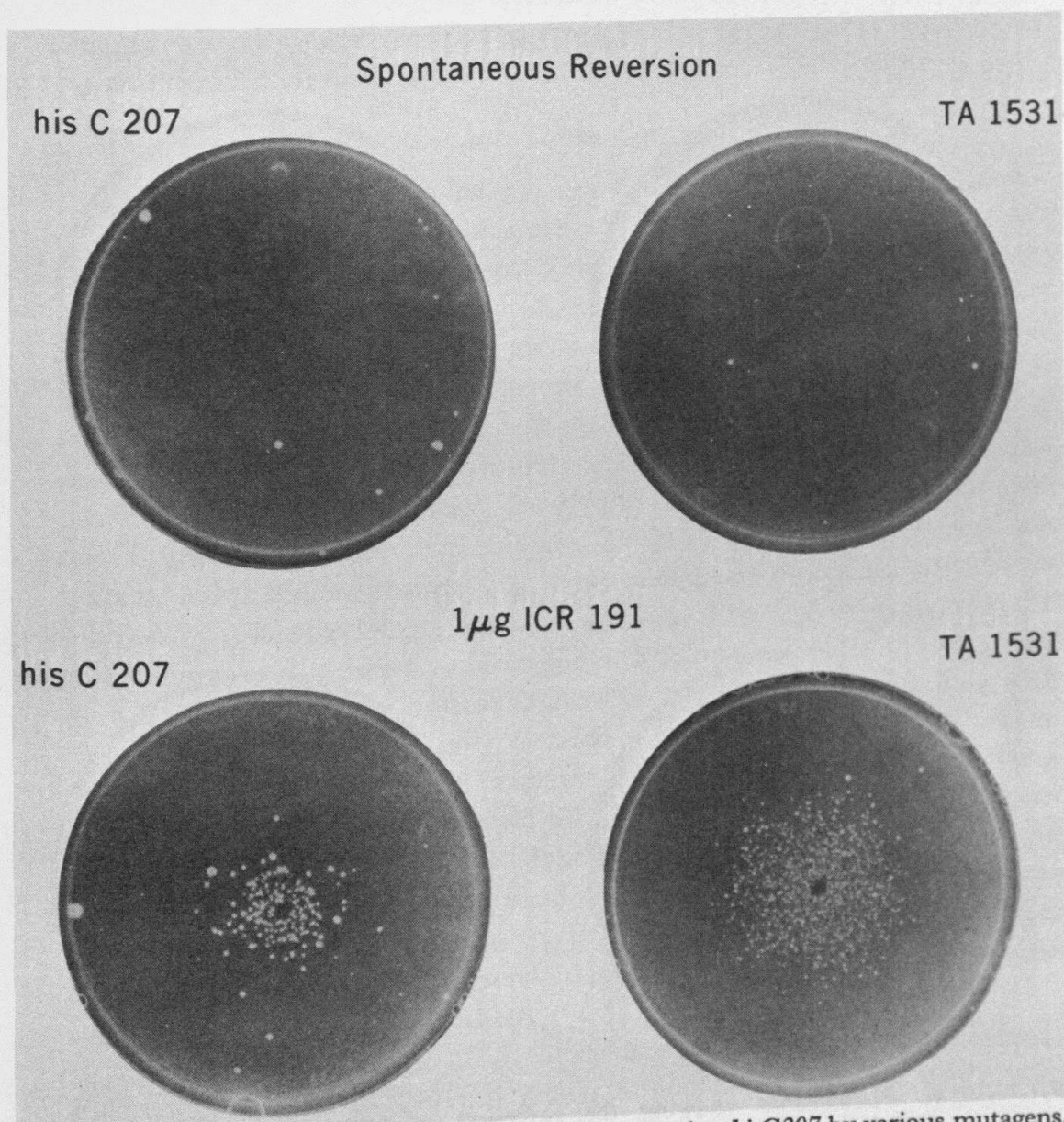


FIGURE 3. Photographs showing the reversion of mutation *hisC207* by various mutagens. The double mutant *hisC207-uvrB* (TA1531) is compared to the *hisC207* strain.

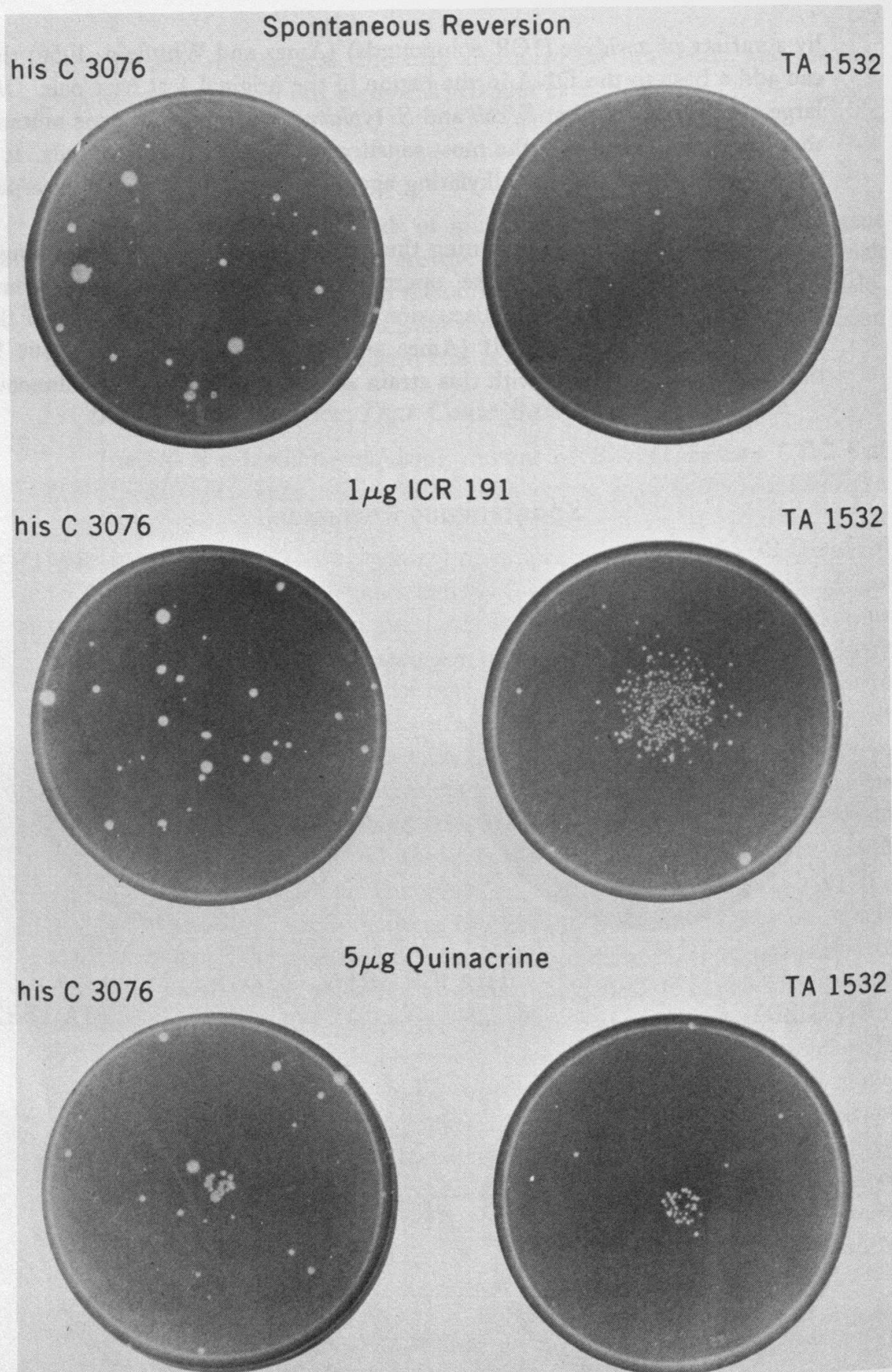


FIGURE 4. Photographs showing the reversion of mutation *hisC3076* by various mutagens. The double mutant *hisC3076-uvrB* (TA1532) is compared to the *hisC3076* strain.

reversion rate of the *hisC207* locus was lower in the TA1531 strain. Figure 3 illustrates some tests with these strains.

b. HisC3076. *HisC3076* is a histidine-requiring mutant that appears to have an added base pair in the aminotransferase gene in *Salmonella* (Oeschger and Hartman, 1970). It reverts spontaneously at a low rate, it is reverted by ICR191 and the other ICR acridines, and it is also reverted by quinacrine (atabrine) and 9-aminoacridine. It also is reverted weakly by diethylsulfate, methyl-nitro-nitrosoguanidine and 1,3-propane sultone; there is evidence that these agents can also occasionally delete a base pair (Oeschger and Hartman, 1970; Yourno and Heath, 1969).

TA1532 is a strain containing the *hisC3076* mutation and a galactose-biotin-*uvrB* deletion. It is more sensitive to ICR191, about equally sensitive to quinacrine, and has a lower spontaneous reversion rate than *hisC3076* (Fig. 4). It is also more sensitive to reversion by alkylating agents than the parent *hisC3076* strain.

3. Detection of Mutagens That Cause Large Deletions of the DNA

The ordinary tester strains will not detect an agent that causes deletions. We have developed a test system that retains the advantages of the positive selection on petri plates and that is specific for agents that cause deletions (Alper and Ames, unpublished results). We have chosen conditions so that two closely linked genes, nitrate reductase and galactokinase, must be deleted in order to have the bacteria grow. The principle of the assay is as follows: Chlorate inhibits *E. coli* and *S. typhimurium* under anaerobic conditions, as it is converted by nitrate reductase to chlorite, which kills the bacteria. Mutations that destroy the nitrate reductase gene enable the bacteria to grow in the presence of chlorate. Galactose kills a galactose epimerase mutant, and mutations that destroy the galactokinase gene eliminate this inhibition by galactose. Single deletions are known that go through both the galactokinase and nitrate reductase genes. Such deletions are the only way for a galactose epimerase mutant to grow on a galactose-glycerol plate with chlorate incubated anaerobically, as both genes have to be mutated for the organism to grow and double point mutations are extremely rare. Thus a crystal of some chemical can be put on a petri plate with a lawn of 10^8 bacteria, and, if the chemical causes deletions, then a ring of mutants should grow up around the crystal after incubation. It would be possible to test the mutants to determine that they are truly deletions and not double point mutants by checking their reversion to growth on nitrate as a nitrogen source under anaerobic conditions.

An epimerase mutant of *S. typhimurium*, *galE503* (formerly *gal-m1*; Fukasawa and Nikaido, 1961), is grown on nutrient broth. One-tenth milliliter of this culture is added to top agar (section III) and poured on a minimal-2% glycerol plate containing 0.1% galactose, 0.3% potassium chlorate,

and 0.1 μ mole of biotin. The plates are incubated aerobically for 8 hr at 37°C, overnight at 37°C anaerobically, and then aerobically for another 48 hr. The mutagens are added to the surface of the plate before incubation as in the other test systems. There are usually about ten spontaneous mutants per plate.

E. A General Test for Mutagenesis

Streptomycin resistance is generally used as a test for mutagenesis in that the streptomycin-resistant mutants can grow on a streptomycin plate while the wild-type bacteria can not. This is not a general test of mutagenesis, however, as the streptomycin-resistance mutation is a mutation in a gene for a ribosomal protein and only certain mutations will give the appropriate altered protein—a complete nonfunctional protein would be lethal for the cell (Silengo *et al.*, 1967). Thus nonsense mutations (UAA, UGA, UAG), frameshift mutations, and deletions are not detected in the streptomycin test.

A more general test is that of mutation to resistance to L-azetidine carboxylic acid (Calbiochem, Los Angeles, Calif.). Azetidine carboxylic acid (AC) is a proline analog that kills bacteria because it is incorporated into protein instead of proline. Mutants resistant to AC appear to be missing a functional proline permease, as they will not grow on proline as a nitrogen source. Any mutation that destroys the functioning of the permease gene, then, will result in a colony being formed by the AC-resistant mutant.

The test has not proved satisfactory for adding crystals of mutagens to plates as in the other tests. It can be used for testing a culture that has been mutagenized in liquid culture to see the increase in AC-resistant mutants over the unmutagenized culture. We ordinarily use wild-type *S. typhimurium* LT-2, but strain TA1530 can be used if an excess of histidine and biotin is added to the plate and the culture. A several-hundredfold increase over the spontaneous rate is obtained either by treating the bacteria with an alkylating agent such as methyl-nitro-nitrosoguanidine or by using an agent such as ICR191, which specifically causes frameshift mutations.

The mutagenized and untreated cultures are compared by making pour plates (see section III) on minimal medium and then putting a 6-mm disk of filter paper containing 3 μ moles of AC on the surface of each plate. The plates are incubated for 2 days and the number of colonies from resistant mutants counted within the zone of inhibition. The numbers are corrected for any difference in the number of bacteria plated. With a well-mutagenized culture it is convenient to plate only 10 μ l instead of 0.1 ml. Alternatively, 15 μ moles of AC may be incorporated into the minimal agar, instead of using a disk of AC.

F. Agents That Have Been Shown to Be Mutagenic Using These Strains

Radiation: ultraviolet,* fast neutrons, X-rays.*

Methylating agents: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine,* streptozotocin,* methyl methanesulfonate,* *N*-nitroso-*N*-methylurethane,* methylazoxymethanol.*

Ethylating agents: diethylsulfate, ethyl methanesulfonate.

Other alkylating agents: β -propiolactone,* β -butyrolactone,* 1,3-propane sultone,* nitrogen mustard,* chloroethylamine,* dibromoethane (ethylenedibromide), ethyleneimine.*

Nonalkylating agents: hydrazine,* hydroxylamine, nitrous acid.

Acridines and acridine-like compounds: quinacrine (atabrine), 9-aminoacridine, and a variety of acridines, aza-acridines, and benzacridines:

Base analogs: 2-aminopurine, 5-bromouracil.

G. Phenotypic Curing

Streptomycin, kanamycin, and neomycin cause phenotypic curing by causing occasional misreading of the messenger RNA (Davies *et al.*, 1965). If a few milligrams of streptomycin is put down on one of the pour plates of the tester strains, a zone of inhibition is observed after incubation. In *hisG46*, but not in *hisC207*, a ring of dense growth peripheral to the zone of inhibition indicates phenotypic curing (Whitfield *et al.*, 1966). The mis-coding results in the occasional misreading of the UAA codon (at the point of mutation) and thus every bacterium can make a minimal amount of functional enzyme and grow at some concentration of streptomycin just below the inhibitory concentration. We have also observed this phenomenon with hydrazine. It may be of interest to know which of the compounds being tested as mutagens can also cause miscoding in protein synthesis. This information is apparent on the tester plates with no extra work. The frame-shift mutant *hisC207* serves as a control, as phenotypic curing is not observed with mutants having an insertion or deletion of a base pair.

H. Testing of Compounds, Availability of Strains, and Improvements of Procedures

I will be glad to mail the strains to people desiring them and to serve as a clearinghouse for new and improved bacterial tester strains. I will also

* The asterisk indicates agents known to be carcinogenic.

keep a list of the response of compounds if investigators will send me their results.

III. GENERAL METHODS

A. Growth of Bacterial Cultures

The various mutants are grown up at 37°C with shaking in nutrient broth. We usually grow up the cultures in 5 ml in a 18-mm test tube with a Bellco plastic closure on top in a New Brunswick rotary shaker. The cultures will grow up overnight and then can be stored in the refrigerator for several weeks. Permanent cultures of the strains are kept as stabs in small screw-cap vials of nutrient broth containing 0.6% agar. The threads of the vials are dipped in molten paraffin before the cap is screwed on, which seals the tube and prevents drying. These stabs can be kept for many years at room temperature.

The number of spontaneous revertants in the cultures affects the sensitivity of the tests to some extent, especially with strain TA1530. It is possible to lower the number of spontaneous mutants in a simple way. Due to the random time of appearance of spontaneous mutants, there is a considerable variation in the number of revertants if a number of parallel cultures are grown up from small inocula. We streak out the culture of the histidine mutant on a nutrient broth plate, and make nutrient broth cultures from five to ten single colonies when they are small. Each culture is then tested to see how many revertants are present, and only the culture with the fewest revertants is saved. Fresh cultures can be made every few weeks by inoculating several parallel cultures with a small aliquot containing no revertants and repeating the process. A fully grown nutrient broth culture should have about 3×10^9 bacteria per milliliter.

We routinely use plugged pipettes and autoclave all materials after use, even though the *S. typhimurium* LT-2 wild-type strain is not highly pathogenic. This strain has been used by many laboratories for many years and also in many class experiments. There has been only an occasional case of diarrhea when someone swallows a mouthful. Those tester strains with a deletion through the galactose operon are not pathogenic, because of their inability to make the lipopolysaccharide endotoxin which contains galactose.

B. Pour Plates for Testing Mutagens

In order to obtain a uniform lawn of the histidine-requiring bacteria (or other tester strains) on a minimal plate, we add 0.1 ml of the nutrient broth culture to a tube (13 by 100 mm) with 2 ml of molten 0.6% agar

containing 0.5% NaCl (top agar) kept at 45°C. This temperature will keep the top agar molten and not kill bacteria. The tube is then mixed by a brief rotation between the palms and poured onto the center of a petri plate of minimal agar medium (we use the Vogel-Bonner, 1956, minimal medium with 1.5% agar and 2.0% glucose). The plate is then jiggled quickly to cover the surface with the liquid and put on the bench to harden—the mixing and jiggling operation should take no more than 10 sec; otherwise, the soft agar starts to harden and the layer is not smooth. The plates can be prewarmed if the air temperature is low.

A trace (0.1 μ mole) of histidine, and an excess of biotin (0.1 μ mole), is added to the plate or to the top agar, so that all the bacteria can grow slightly to make a background lawn, so that any inhibition caused by the compounds can be seen. This addition is essential, as growth of the cells is required for mutagenesis in the case of many of the mutagens.

The mutagen may be added to the plate any time after the top agar has hardened (usually 5 min is sufficient). The addition can be delayed for several hours. It is usually convenient to put some crystals (1 to 5 mg) of the mutagen as a point near the edge of the plate with a broken applicator stick that is used as a spatula. If the compound is a liquid, about 5 μ l is usually used; this will soak into the agar in a short time. A control of the spontaneous mutation rate should also be run for each tester strain on a separate plate. Plates are incubated upside down for 2 days at 37°C.

If a large number of compounds are being tested, then four or more compounds can be put on the same plate. If any of them inhibits the whole plate, or gives a large number of mutants, then the compounds must be retested individually.

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NOTE ADDED IN PROOF

1. In Fig. 1, 2 mg of sodium nitrite was used.
2. In the assay for deletions the ingredients in the agar plate should be autoclaved separately. The top agar should have 0.1 ml of 4% glucose added to it. This increases sensitivity by allowing time for gene expression before the galactose kills the cells. The plate should contain no glucose other than this. When this is done about fifty spontaneous mutants are observed per plate.
3. Hycanthone and nitroquinoline-*N*-oxide* should be added to section F. They specifically cause frame shifts (Hartman *et al.*, *Science*, in press) and can be detected with TA 1532 and *hisD3052* respectively.
4. The carcinogen aminofluorene and a variety of its derivatives, especially nitrosofluorene, cause frame shift mutations and can be detected with strain TA 1533 (*hisD3052* lacking *uvrB*).

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CHEMICAL MUTAGENS

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