BACTERIAL MUTAGENICITY TEST:
THE AMES Salmonella PLATE INCORPORATION ASSAY.

Introduction

Many carcinogens are also mutagens. One widely-utilized, relatively cheap, rapid and simple means of determining whether a chemical can cause mutations (alterations in genetic material) is the Ames plate incorporation assay. For more details see Ames et al., 1975, Mutation Research, Vol. 31, pp. 347-363. This assay procedure utilizes a specially-constructed type of bacteria (variants of *Salmonella typhimurium*) which has already undergone a mutation to render it incapable of synthesizing its own histidine as normal bacteria can. For growth, these bacteria therefore need to be provided with exogenous histidine. On exposure to "mutagenic" agents, these bacteria will mutate back (revert) to the wild-type, and regain the ability to make histidine. Therefore we can expose these bacteria to a chemical, and culture the exposed bacteria on plates containing minimal histidine, so that only those organisms which have undergone the back-mutation or reversion will be able to grow, and the unmutated ones will starve for lack of histidine. The larger the number of bacterial colonies formed, the more mutagenic the compound. Some chemicals can induce mutations all by themselves or after undergoing metabolic activation by the bacterial enzymes. These are the "direct-acting" mutagens. Other compounds, typically polycyclic aromatic hydrocarbons (PAH), require metabolism by the cytochrome P-450 system to be active (often via generation of an epoxide). These are termed "indirect-acting" mutagens. We therefore test compounds both without and with an enzyme preparation containing cytochrome P-450. This is the 9000 x g supernatant from the livers of rats treated with the inducing agent Aroclor 1254, and is termed S9.

We will examine the mutagenicity of environmental complex mixtures in one strain of *Salmonella typhimurium*, TA98, with and without S9. This variant responds preferentially to frameshift-inducing mutagens, in contrast to other variants such as TA100 which responds more readily to agents which cause base-pair substitutions.

Safety: The Salmonella strains used in this assay have undergone modifications to their cell wall structure and DNA repair systems such in principle they cannot survive long outside their culture media. However the genus and species from which they are derived are notorious human intestinal pathogens, and these organisms should therefore be treated with respect and care.

Likewise the compounds provided for assay are not considered to be human carcinogens, but the mere fact that they may be able to cause genetic damage to bacteria indicates that they should be handled cautiously: Wear lab coats, disposable gloves, do not pipette by mouth, do not touch chemicals or bacteria with naked skin, wipe up all spills promptly.
Good housekeeping (e.g. leaving outdoor clothing, backpacks, inessentials, etc. well out of the way) will also help reduce one problem endemic with this lab class, namely contamination of the plates with airborne agents, particular fungi.

Today’s problem

The EPA is (once again) considering the health effects of automotive emissions. Since you are employed in the Environmental Carcinogenesis Division, you have been tasked with evaluating the potential carcinogenicity of two samples of particulate matter, collected by scraping the soot from the tail-pipes of two automobiles. One is from Diesel-fueled Brand X, the other from Gasoline-fueled Brand Y. Since these samples are not available in large enough quantities for whole-animal carcinogenicity tests, you focus on mutagenicity, and you suggest to your Division Director that you might be able to identify particular chemicals that might be responsible for the majority of the biological activity of emission mixtures. From your reading of the literature you identify two chemicals that might be good candidates: cyclopenta[cd]pyrene and 2-nitrofluorene. Your goal today is to see whether the mutagenicity characteristics of these two compounds match those of the particulate matter samples.

Two of the compounds to be assayed for mutagenic activity are:

1. Neutral red, a dye used in histological studies for staining blood and embryo tissues for histology studies.
2. 2-Nitrofluorene, a polycyclic aromatic hydrocarbon emitted from combustion processes.
3. A mystery compound.

Each group will test both compounds and both extracts at three different dose levels, and also an equal volume of solvent alone (zero dose, or solvent control). The test compounds are provided already made up in solution in dimethylsulfoxide (DMSO), and diluted to the appropriate dose levels.

The doses are:

1. Cyclopenta[cd]pyrene, 0, 5.0, 10, 50 ug/mL
2. 2-Nitrofluorene, 0, 2.5, 5.0, 10 ug/mL
3. Diesel X, Neat extract, dilution 1:10, dilution 1:50
4. Gasoline Y Neat extract, dilution 1:10, dilution 1:50

Each dose level is to be assayed in duplicate.

Procedure

Each of you is provided with

a) an overnight culture of TA98 bacteria in nutrient broth
b) S9 protein suspension made up with an NADPH-generating co-factor system
(c) 13 x 100 tubes containing 2 mL of molten top agar in water-bath
(d) minimal medium plates
(e) test compounds, diluted to three dose levels
(f) solvents
(g) sterile pipettes

Bacteria, test compound and S9 (where needed) are added to 2 mL of molten top agar (in 13 x 100 mm test tubes), as shown in Table 1.

Table 1

<table>
<thead>
<tr>
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<th>Without S9</th>
<th>With S9</th>
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<tbody>
<tr>
<td>Solvent Controls</td>
<td>0.1 mL bacterial broth</td>
<td>0.1 mL bacterial broth</td>
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<tr>
<td></td>
<td>0.1 mL solvent (DMSO)</td>
<td>0.1 mL solvent (DMSO)</td>
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<tr>
<td></td>
<td>0.5 mL S9 mix</td>
<td>0.5 mL S9 mix</td>
</tr>
<tr>
<td>Test Compound</td>
<td>0.1 mL bacterial broth</td>
<td>0.1 mL bacterial broth</td>
</tr>
<tr>
<td></td>
<td>0.1 mL dose solution</td>
<td>0.1 mL dose solution</td>
</tr>
<tr>
<td></td>
<td>0.5 mL S9 mix</td>
<td>0.5 mL S9 mix</td>
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The contents of the tube are mixed quickly by vortexing then poured out onto an agar plate. Tilt the plate to spread the mixture evenly, place aside to allow the agar to set.

Each dose level is to be assayed in duplicate. LABEL THE PLATES.

When all the plates have been poured and the agar has set, move them into the incubators, place them on the shelves upside down and no more than three deep, and leave them there for 48 h while the bacterial colonies grow. After 48 h, i.e. on Friday afternoon, remove plates from the incubators, place the plates in the refrigerator (still upside down) and count the bacterial colonies on Wednesday Oct 12th.

Results

Count the colonies (revertants to histidine independance, or prototrophy) on each plate. Record the counts on the blackboard. Combine results from the whole class for your write-up.

Data Interpretation

The data obtained from Ames assays can be handled in a number of different ways.

The generally-accepted basic criteria for determining whether a compound is mutagenic (positive) or not (negative) are (1) plate counts at least double the background count, and (2) a dose-related increase in plate counts. Ideally both of these criteria should be met, but in special circumstances either one may be acceptable.
(1) Mutagenicity index:

Mutagenicity index = \[ \frac{\text{Colony counts on the test plate}}{\text{Average counts on the control plates}} \]

A ratio \( \geq 2 \) is generally considered positive, < 2 is negative.

(2) Does the plate count rise with increasing dose?
Yes = positive, No = negative.

Beyond simple determination of positive/negative, there is a need for expressing the level of activity so that numerical comparisons can be made from one compound or set of test conditions to another.

This can be accomplished by in a number of ways.

**Specific Activity.** This is the slope of the linear portion of the dose-response curve. It is obtained by graphing the mutagenic response (revertant counts per plate) as the dependent variable, against increasing compound dose per plate as the independent variable. Thus one can express specific activity as revertants per mg (or per \( \mu \)g or per nanomole, as appropriate) of compound.

Often the dose response curve has no good linear portion. In that case the following might be recorded:

Dose needed to double the background rate. This may be evident from the data, or may need to be read off the graph of mutagenic response against dose.

Maximum mutagenic response. The highest number of revertants per plate which a compound is capable of producing.

**Write-up**

Describe the experimental procedures in your own words.

Determine whether each compound is active, with or without S9.

Determine the Specific Activity, Dose needed to double the background rate, and Maximum mutagenic response, for each compound and condition that you consider to be active.

Answer the following questions:

i) Which compounds are positive, and under what conditions?

ii) On which criteria do you base these conclusions?

iii) Which compound(s) is/are direct-acting, and which one(s) require(s) metabolic activation?

iv) Which extract(s) is/are direct-acting, and which one(s) require(s) metabolic activation?

v) What do these results suggest with respect to the genotoxic hazards presented by these emissions to humans?

(vi) Which of the two pure compounds would be a good surrogate for each of the emission extracts?