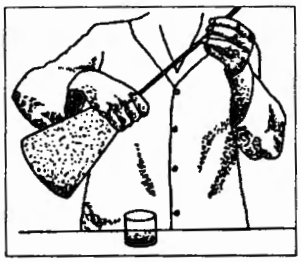
METODI



E.P.A. - 600/2-80-057 March 1980

TOXICITY OF LEACHATES

by J.L. Epler, F.W. Larimer et al.
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Sebbene la recente normativa nazionale relativa allo smaltimento dei rifiuti abbia sancito che la tossicità e la nocività siano valutabili mediante un criterio di presenza/assenza di una serie ampia -ma pur sempre finita- di molecole, riteniamo sia necessario stimolare l' interesse dei gestori dei problemi ambientali sulle potenzialità discriminative di test tossicologici opportunamente congegnati. Un tossico è, per definizione, una sostanza nociva agli esseri viventi ed è fonte di continua sorpresa dover constatare come questo aspetto venga tanto trascurato dal legislatore. Riteniamo utile, quindi, proporre il volume **"Toxicity of leachates"** pubblicato dall' Enviromental Protection Agency nel 1980.

Il volume rappresenta il risultato di una ricerca multidisciplinare tesa a produrre il protocollo di base per la valutazione della tossicità degli estratti da rifiuti solidi di varia tipologia industriale. Nel 1976, infatti, attraverso il Resource Conservation and Recovery Act, l' E.P.A. ha ricevuto l' incarico di proporre procedure di gestione dei rifiuti pericolosi, sviluppando metodologie per caratterizzare ed identificare quei rifiuti che possono rappresentare un potenziale pericolo per la salute umana e per l' ambiente. Il volume, oltre al commento critico delle procedure proposte e dei risultati, presenta le metodologie sperimentali consigliate.

La Redazione ritiene di rendere un servizio utile ai propri lettori proponendo i testi integrali dei metodi biologici ed un breve riassunto della discussione generale. Chi fosse interessato ad ottenere copia fotostatica del testo di capitoli non stampati dovrà rivolgersi alla Segreteria del C.I.S.B.A., specificando al meglio la richiesta in base all' indice del volume.

Con l' obiettivo di produrre un protocollo operativo per giudicare la pericolosità di un rifiuto, e quindi le modalità di smaltimento più idonee, è stato concordato un piano di ricerca da sviluppare seguendo quattro direttrici:

1- sperimentare le tecnologie più idonee per la procedura di estrazione;
2- individuare i parametri chimici e le metodologie più idonee per caratterizzare gli estratti;
3- sperimentare la procedura di estrazione (EP)

su svariati rifiuti industriali per verificare l' applicabilità del metodo;

4- individuare i saggi di laboratorio più idonei per caratterizzare la mutagenicità, la fitotossicità e la tossicità acquatica degli estratti.

Sono stati utilizzati 17 fanghi rappresentativi di varie tipologie di rifiuti industriali (industria alimentare, tessile, galvanica; impianti per la produzione di energia elettrica o di gasificazione, ecc.); essi sono stati giudicati esclusivamente sulla base delle loro caratteristiche individuali rilevate durante il corso delle analisi.

Poichè lo stato fisico dei campioni può variare fortemente -si passa dal polveroso al melmoso- non è stato possibile applicare un' unica procedura di subcampionamento. Durante l' estrazione sono stati registrati il pH iniziale e finale, il volume di acido acetico 0.5 N necessario per portare inizialmente la soluzione a pH 5 e il volume aggiunto nelle 24 ore, e la conducibilità dell' estratto finale; la riproducibilità dell' estrazione è risultata, però, molto bassa. In base agli standard delle acque potabili ed agli elenchi dei principali inquinanti sono sati selezionati i costituenti potenzialmente tossici o mutageni per i quali effettuare la ricerca analitica: nell' elenco sono compresi alcuni elementi chimici, sostanze organiche volatili, organoalogenati e idrocarburi policiclici aromatici.

Per quanto concerne le prove tossicologiche, si è proceduto ad uno screening, con un test a breve termine, seguito da un saggio a lungo termine condotto con diluizioni simulanti condizioni ambientali reali.

La potenziale pericolosità degli estratti nei confronti dell' ecosistema acquatico viene valutata con *Daphnia magna*. Per i test a breve termine è stato stabilito un periodo di esposizione di 48 ore; per i test a lungo termine (28 giorni) è stato scelto di valutare se l' esposizione continua alle diluizioni 1:100 e 1:1000 altera la capacità riproduttiva di *D. magna*; la diluizione 1:1000 è stata ritenuta ragionevolmente protettiva per gli organismi acquatici esposti ai percolati delle discariche di rifiuti. Per l' analisi statistica dei risultati è stata utilizzata l' analisi della varianza combinata con il Multiple Range Test di Duncan. Tutti gli estratti saggiati sono risultati più o meno tossici a breve termine; in due casi la LC_{50}-48h era pari allo 0.0005%, quindi duecento volte inferiore alla diluizione massima utilizzata nel test cronico. In linea di massima, non si sono registrati effetti a seguito dell' esposizione a lungo termine. Il saggio su *Daphnia* si è dimostrato un test sensibile e riproducibile: l' unico problema incontrato è quello relativo all' effetto stimolante la riproduzione determinato da basse concentrazioni di acetato negli estratti.

Anche nel caso dei saggi per la determinazione degli effetti degli estratti sulle piante terrestri è stata seguita la duplice ipotesi di effetti a breve e a lungo termine, nella seconda delle quali i semi del gruppo "trattamento" sono stati posti a contatto con un estratto al 10% per simulare la diluizione che si presume avvenga durante il drenaggio delle discariche verso le acque di falda. Per tener conto delle differenze di sensibilità delle specie vegetali alle sostanze chimiche sono stati utilizzati semi di monocotiledoni e di dicotiledoni di grande importanza agronomica.

Il test di allungamento della radice è risultato un saggio affidabile; bisogna però porre attenzione alle interferenze determinate dall' acido acetico usato nella preparazione dell' estratto. Per i saggi a lungo termine è stata scelta la sabbia come supporto solido: ciò permette di evitare le interferenze sulla fitotossicità esercitate dai vari tipi di suolo; la ricerca si sta però orientando verso la coltura idroponica.

Nel protocollo di individuazione di un potenziale pericolo ambientale non possono mancare saggi per la determinazione di proprietà oncogene, mutagene e teratogene delle miscele complesse presenti nell' estratto dei fanghi. Nel protocollo E.P.A. sono perciò previsti saggi indicatori di mutagenicità e cancerogenità; la batteria di saggi deve sempre essere eseguita al completo per ovviare le differenze "sistemiche" di reattività delle sostanze. Essa permette di rilevare agenti diretti e indiretti sia di mutazioni puntiformi che di danni generici al DNA.

Lo sviluppo di un protocollo di screening per sostanze potenzialmente pericolose è reso complicato dal fatto che dovrebbero essere utilizzzate un gran numero di specie, data la diversa sensibilità di ciascuna di esse. Nonostante ciò, perfino con protocolli che utilizzano un numero limitato di specie e di condizioni, come in quello presentato, le molecole pericolose possono essere individuate e sottoposte ad ulteriori controlli. E' ragionevole, infatti, supporre che le sostanze che forniscono risultati significativamente diversi dai controlli in almeno un test di tossicità siano potenzialmente pericolose: il loro destino ambientale merita, quindi, di essere seguito più attentamente.

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CONTENTS

APPENDIX A

EXTRACTION PROCEDURE (EP)

(A) EQUIPMENT

(I) An agitator which, while preventing stratification of sample and extraction fluid, also insures that all sample surfaces are continuously brought into contact with well-mixed extraction fluid.

(II) Equipment suitable for maintaining the pH of the extraction medium at a selected value.

(B) PROCEDURE

(I) Take a representative sample (minimum size 100 g) of the waste to be tested. Separate sample into liquid and solid phases. The solid phase is defined as that fraction which does not pass through a 0.45-μm filter medium under the influence of either pressure, vacuum, or centrifugal force. Reserve the liquid fraction under refrigeration (1-5°C) for further use.

(II) The solid portion of the sample, resulting from the separation procedure above or the waste itself (if it is already dry), shall be prepared either by grinding to pass through a 9.5-mm (3/8 in.) standard sieve or by subjecting it to the structural integrity procedure.

(III) Add the solid material from paragraph II to 16 times its weight of deionized water. This water should include any water used during transfer operations. Begin agitation and extract the solid for 24 ± 0.5 h. Adjust the solution to pH 5 and maintain that pH during the course of the extraction using 0.5 N acetic acid. If more than 4 ml of acid for each g of solid would be required to maintain the pH at 5, then once 4 ml per g of solid has been added, complete the 24-h extraction without adding any additional acid. Maintain the sample between 20-40°C during extraction.

(IV) At the end of 24-h extraction period, separate the sample into solid and liquid phases as in paragraph I. Adjust the liquid phase with deionized water so that its volume is 20 times that occupied by a quantity of water at 4°C equal in weight to the initial sample of solid (e.g., for an initial sample of 1 g, dilute to 20 ml). Combine this liquid with the original liquid phase of the waste. This combined liquid, including precipitate which later forms from it, is the Extraction Procedure extract.

APPENDIX D

MATERIALS AND METHODS FOR THE AQUATIC TOXICITY SCREENING TESTS

The tests, in order of occurrence, were: (1) A preliminary 48-h acute toxicity determination; a few test concentrations over a wide range. (2) A definitive 48-h acute toxicity test; test concentrations over a narrower range that in (1). (3) A 28-day life-cicle, chronic toxicity test; two diluitions (1:100 and 1:1000) of the extract. (4) A final 48-h LC_{50} determination (this determined if the toxicity of the extract had changed during the 28-day life-cicle test).

ACUTE TOXICITY TESTS

Laboratory-cultured, first-instar *Daphnia magna*, less than 24 h old, were the test animals. Five organisms were exposed to 80 ml of extract solution in covered 100-ml glass beakers. Temperature was maintained at 20 ± 0.5°C in an environmental chamber with alternate light/dark periods of 12 h each. The dilution water used was well water with a pH of 7.8, an alkalinity of 119 mg/l, and a hardness of 140 mg/l. The extracts were neutralized to ph 7.0 with NaOH. The pH of the extract diluitions was measured at the beginning and conclusion of each test.

Serial geometric diluitions with well water were made for each extract. The concentration of each extract solution was 60% of the preceding one. The range of diluitions was select to bracket 48-h LC_{50} values obtained from preliminary toxicity tests. Controls consisted of animals exposed to: (1) well water without extract, or (2) neutralized hydrochloric or acetic acid equal to highest concentration of acid used in the acute toxicity test. All tests were done in triplicate. The range of diluitions was select to bracket 48-h LC_{50} values predicted from preliminary toxicity determinations. Additional control beakers were included containing a concentration of neutralized acetic acid equal to the highest concentration used in the acute toxicity tests. Control beakers of diluition water without added extract were also included. Values for 48-h LC_{50} and 95% fiducial intervals were obtained by computerized PROBIT analytical procedures.

CHRONIC TOXICITY TESTS

The test animals were first-instar *D. magna* less than 24 h old. They were exposed individually to 50 ml of either 1:100 or 1:1000 diluition of the extract in covered 100-ml beakers. Temperature and lighting conditions were the same as for the acute test. In each test, 40 animals were used as follow: 10 were exposed to each test diluition, 10 to well water only, and 10 to neutralized acetic or hydrochloric acid at the same concentration as used in the 1:100 diluition (acid was used in the EP, therefore an appropriate control was necessary). The animals were transferred to freshly prepared test solutions three times a week, and at those times they were fed 2 mg of prepared trout chow. At the time of transfer, the number of young and number of broods present in each beaker were counted. The pH of the test solutions was measured at the beginning and end of each test. The tests lasted 28 days, or less if all animals had died before that time.

APPENDIX E

RADICLE LENGHT ASSAY

Two level of tests were completed on 17 solid waste extracts by use of the EP. Of these, 11 were from coal processing plants and 6 were from various types of industrial plants. One test was run on arsenic-contaminated groudwater taken from a well near a disposal site. Level one studies consisted of a root elongation bioassay of radish (*Raphanus sativus* L. c.v. Early Scarlet Globe) and sorghum (*Sorghum vulgare* var. saccharatum c.v. Sugar Drip) seeds. In previous tests, seeds were germinated in petri dishes and root (radicle) lengths of treatment and controls were compared. However, the time required to measure root lengths was so great due to their coiled growth pattern that it was not practical to use enough seeds for good statistical comparisons. Therefore, we developed special vertical germination chambers which took advantage of the geotropic growth response of plants, resulting in straight hypocotyl growth and a tenfold reduction in measurement time.

One approach to reduce variability was to sieve seeds to separate them into size categories. U.S.A. standard testing sieves numbers 8, 10 and 12 openings (in mm) of 2.36, 2.00, and 1.70, respectively, were

used for separation. Within a test only one seed size was used for controls and test diluitions. Although 200 seeds were used for each treatment, only 150 seeds were actually measured. The excess allowed for exclusion of nongerminating seeds and for radicles which were less than 5 mm long.

The germination chambers were constructed of 3-mm-thick Plexiglas (Figure E-1) with inside dimensions of 10 cm high x 1.5 cm wide x 71 cm long. The size of the chambers was determined by the size of the incubator in which they were to be used. Chambers were mounted on a Plexiglas base support. Two pieces of 3-mm-thick Plexiglas were cut to an appropriate size to fit inside a chamber but extended above the chamber sides about 3 cm for convenience in handling. One hundred depressions (drilled with an electric drill and bit) spaced at 2-cm intervals in staggered rows 2 cm apart across one of the Plexiglas sheets served as seed counters, seed spacers, and to help hold the seeds in place. Seeds were placed on the Plexiglas sheet and brushed into the depression. A piece of blotter paper was saturated with the solution to be tested and pressed firmly against the seeds until impressions were seen. Additional test solution, up to a total of 100 ml for 48-h tests and 125 ml for 72-h tests, was added to the germination chambers. We recommend that initially the blotter paper be saturated with the extract in a flat tray rather than standing on edge in the chambers, since standing on edge could result in differential movement of chemicals up the paper causing chromatographic separation and variable doses to seeds at different positions. The second Plexiglas sheet was positioned so that the seeds and blotter paper were sanwiched in between the two sheets of Plexiglas, which were than taped securely on the sides and top and placed vertically into the chamber. A Plexiglas lid was placed on top to reduce evaporation.

The entire apparatus was then placed in an unlighted incubator set at 25°C. A small fan was installed in the growth chamber to exhaust volatiles after some tests had already been made. The fan affected the chamber temperature, thus thermostat adjustments were necessary. During this period of adjustment, tests continued, to avoid delay. Since controls are run with each test, we were not concerned about effects of these temperature differences between tests. We used two chambers (200 seeds) for each test solution or for each concentration of a particular solution and two chambers containing distilled water as controls. After a predetermined time period (48 h for radish, 72 h for sorghum) the chambers were removed from the incubator and the root lengths were measured with calipers.

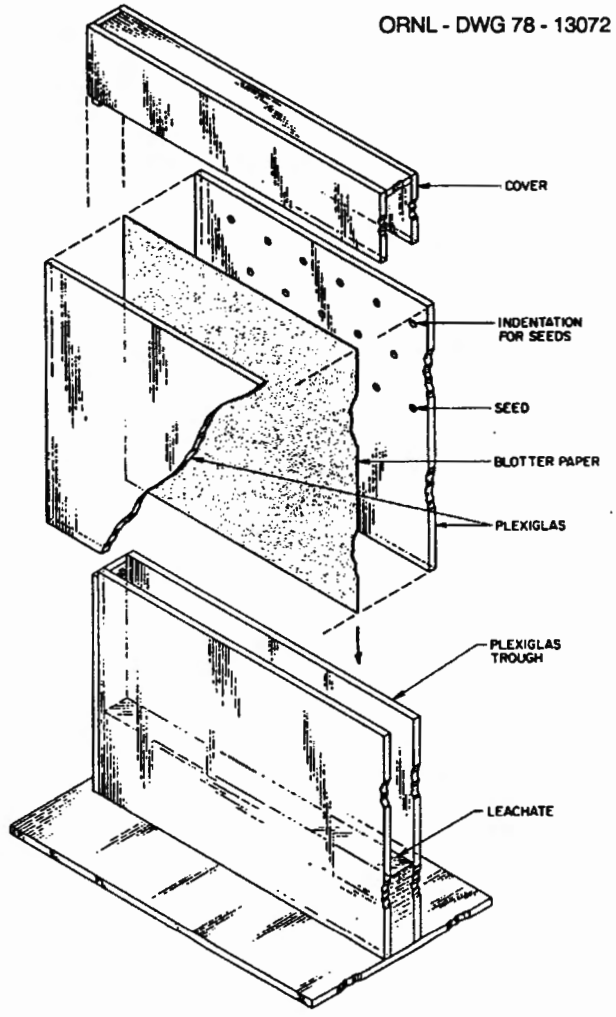

Figure E-1. Germination chamber.

For cleaning, the chambers were filled with an appropriate cleaning solution (0.1 *N* HCl) and allowed to stand until their next use, when they were rinsed with distilled water. The rest of apparatus was washed with two pipette washers, one containing 1 *N* HCl and one connected to a distilled water supply for rinsing.

Acetic acid was used in the EP to maintain the pH of the extract at approximately pH 5.0. Since acetic acid is toxic to plants, the highest concentration of extract used in the root elongation test was the

concentration having less than 5.5 ml/l of 0.5 *N* acetic acid. In preliminary tests this and higher concentrations of the organic acid were toxic to radish, sorghum, wheat, and soybean seeds (Table 23).

The material referred to as arsenic-contaminated groundwater sample was not carried through the EP, but was diluted directly from the original solution for the root elongation tests. Since this particular waste was extremely toxic (based on an initial test with radish seeds) and safety problems were not yet resolved, further study (greenhouse testing) was not undertaken.

APPENDIX F

SEEDLING GROWTH ASSAY

The long-term seedling growth studies were conducted with wheat (*Triticum aestivum* c.v. Bear) and soybean (*Glycine max* c.v. Centennial). Soybean plants were grown in 1 l of sand and wheat in 1.5 l. To 1 l of sand (sand which passes through a 25-mesh sieve) was added 350 ml of a 10% concentration of a solution containing the EP extract plus plant nutrients (20-20-20 N-P-K, plus micronutrients, one tablespoon per gallon of solution; Ralston-Purina, St. Louis, Mo.). There were 50 wheat seeds in each of 5 containers and 15 soybean seeds in each of 10 containers, giving a total of 250 and 150 seeds, respectively.

Sand was selected as a growth medium to eliminate potential confounding of test results by attenuation of toxicants associated with clay particles and organic matter present in natural field soils. Establishment of a more realistic soil medium capable of being universally standardized for application to such assays was deemed to be a research task in itself beyond the scope of this project.

Plants were exposed to the solution above added to sand and misted with an atomizer for leaf exposure. The dose was sufficient to restore loss by evapotranspiration. The amount of time between each application ranged from every other day to every 3 days.

Wheat plants were grown for 2 weeks and soybeans for 3 weeks. At harvest, sand was washed from the roots, roots and shoots were separated, and dry weights were recorded for 12 of the extracts. Five soybean plants and ten wheat plants were consolidated to reduce variability between samples. The N value was the number of sample group available. A standard t-test was used for comparison of treated and control weights. The last four extracts (fluidized bed residue, municipal sewage sludge, power plant No. 2 fly ash, and power plant No. 1 treated scrubber sludge) were compared with controls by measurement of lengths of root and shoot. Measuring length was much faster, and the N value was larger since each plant represented one observation. Also, it was always possible that not all sand was completely rinsed off of the roots, which would create an error in final weight; length measurements eliminate this problem.

APPENDIX G

Salmonella MUTAGENICITY ASSAY

The realization that the list of potential chemical carcinogens is growing faster than our capacity to test the materials and the enormous increase in industrial and technological activities have created an interest in short-term test procedures for the identification of genetic hazards associated with environmental chemical polluants. Although the health effects of chemicals in the environment are being extensively studied, it is obvious that short-term procedures are necessary to reduce the study time for evaluating the large number of potentially hazardous substances. To control the problem of environmental

carcinogenesis, greater number of these compounds are to be screened and assigned priorities for further testing. This appears to be the primary role of the short-term test. Not only should a meaningful short-term test be faster, easier to interpret, more sensitive, and less expensive, but it must also be reliable and relevant to the in vivo assays.

Among the various short-term assays which utilize microbial organisms, the *Salmonella* test system developed by Ames has been widely used as a prescreen for the determination of genetic and potential carcinogenic hazards of complex environmental effluents or products. This test system has been examined more extensively than any other short-term assay for correlating mutagenicity and carcinogenicity.[1] It utilizes a series of histidine-requiring mutants that revert after treatment with mutagens to the wild-type state (histidine independent). Generalized testing of the compound is accomplished by use of three strains (TA1537, TA1538, and TA98) that detect frameshift mutagens and two strains (TA1535 and TA100) that detect base-pair substitution mutagens. The design of the test is shown in Figure G-1.

A recommended protocol outlining the preparation of the components of this test has been published by Ames et Al.[2] Some chemicals like dimethylnitrosamine, certain hydrazines, and volatile liquids which are not mutagenic in the standard plate assay are active in the modified procedure, designated the

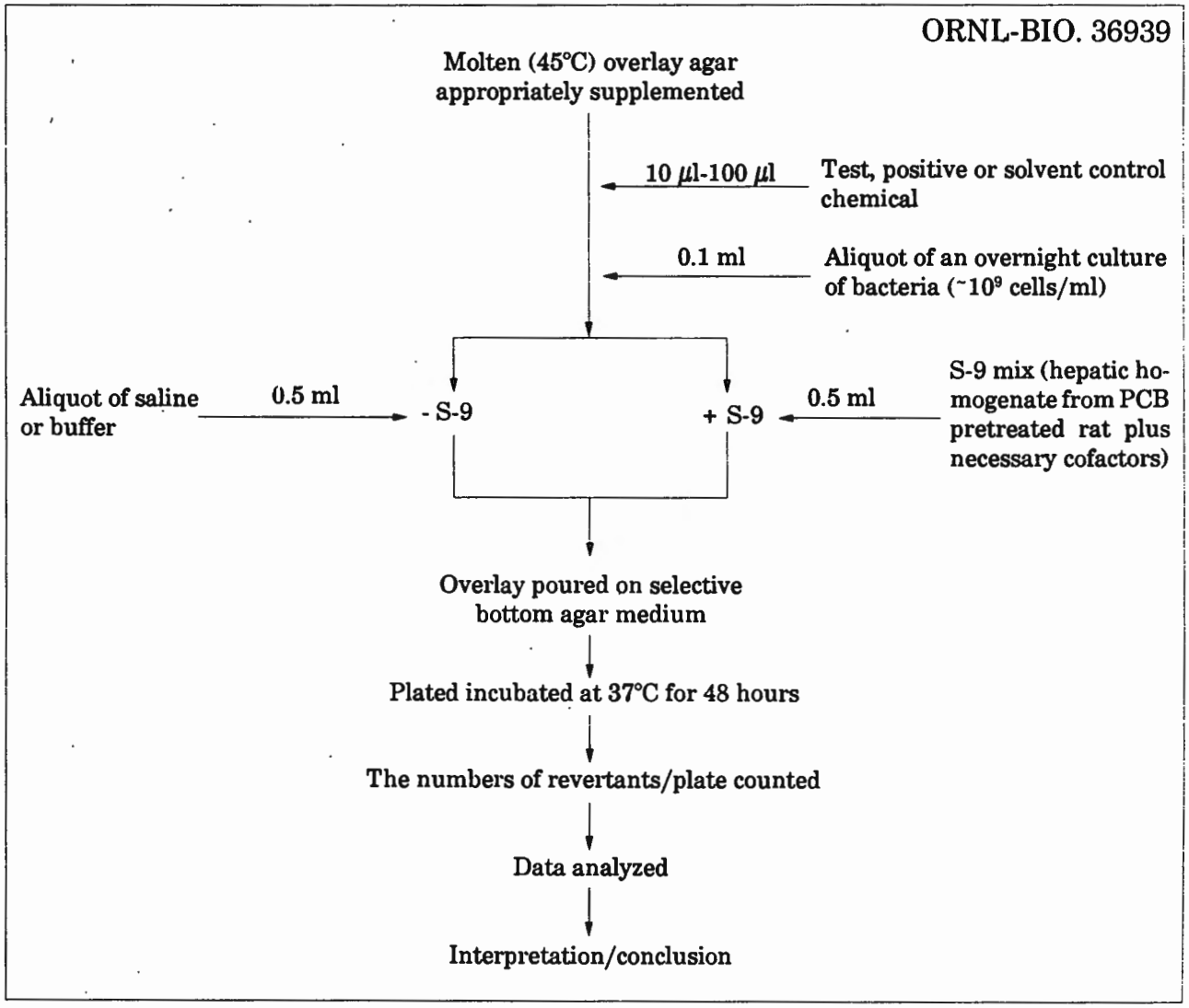

Figure G-1. Reverse mutation assay (agar incorporation method).

preincubation technique. This modified procedure detects not only these compounds, but also the majority of the compounds that have been shown to be active in the standard plate assay.

BACTERIAL STRAINS

Four *Salmonella typhimurium* indicator strains, TA1535, TA1537, TA98, and TA100, are recommended for screening purposes. TA1535 and TA100 have base-pair substitution mutation in the histidine operon; TA100 also contains an R factor which renders the strain more sensitive to certain mutagens, possibly through error-prone repair. TA1537 and TA98 have frameshift mutation in the histidine operon; TA98 contains an R factor and is more sensitive than TA1538. TA1537 is recommended because its unique sensitivity to some agents like 9-aminoacridine and certain ICR compounds. The characteristics of these strains are shown in Table G-1.

TABLE G-1. PROPOSED BACTERIA STRAINS*

Strain designation	Gene affected	Additional mutations		
		Repair	LPS	R factor
TA1535	*hisG*	*uvrB*	*rfa*	-
TA1537	*hisC*	*uvrB*	*rfa*	-
TA98	*hisD*	*uvrB*	*rfa*	pKM101
TA100	*hisG*	*uvrB*	*rfa*	pKM101

* See Ames et al.[1] for references.

STORAGE AND CHECKING OF TESTER STRAINS

All strains are initially grown in nutrient broth (8g Difco-Bacto nutrient broth, 5g NaCl/l) at 37°C for 16 h. The strains are checked for the genetic markers in the following ways:

Histidine Requirement. Streak the cultures on minimal plates both with and without histidine (spread 0.1 ml of sterile 0.1 *M* L-histidine on the agar surface). Biotin (0.1 ml of 0.5 *mM* per plate) is also essential for these strains. The strains should grow on plates containing both histidine and biotin.

Deep Rough Character. A sterile filter paper disc containing crystal violet (10 μl of 1 mg/ml) is placed on a nutrient agar petri dish containing 0.1 ml (about 10^8 bacteria) of the nutrient broth culture to be tested in a thin overlay of top agar. After 12 h incubation at 37°C, a clear zone of inhibition around the disc (about 14- to 18-mm diameter) indicates the presence of *rfa* mutation.

Presence of Plasmid. The strains with R factor (TA100 and TA98) should be checked routinely for the presence of the ampicillin resistance. Streak a small amount (10 μl of 8 mg/ml in 0.02 *N* NaOH) of an ampicillin solution across the surface of a nutrient agar plate. After the streak is dry, cultures to be checked are cross-streaked against the ampicillin, and after incubation for 12-24 h at 37°C, strains which do not contain the R factor will show a zone of growth inhibition around the ampicillin streak, whereas strains containing R factors will not.

Storage. Frozen permanent cultures containing fresh nutrient broth cultures (0.8 ml) with dimethylsulfoxide (0.07 ml) are prepared and maintained in a Revco freezer at -80°C. A working source of these cultures is maintained on master plates which are prepared as follows:

0.1 ml of sterile 0.1 *M* L-histidine is spread on the surface of a minimal glucose agar plate. After the histidine solution is absorbed by the agar, 0.1 ml of sterile 0.5 *mM* biotin is added in the same way. For TA98 and TA100, 0.1 ml of an 8 mg/ml ampicillin solution (in 0.02 *N* NaOH) is added. By use of a sterile loop, nutrient broth culture of the tester strain is streaked across the agar (for TA98 and TA100, plates with ampicillin are used) and incubated at 37°C for 24 h. These master plates with the cultures are stored at 4°C and can be used for several months to grow working cultures.

PREPARATION OF RAT LIVER S-9

Male Sprague-Dawley rats (of about 180-200 g weight) are given a single intraperitoneal injection of Ar-1254 at a dosage of 500 mg/kg (vehicle, corn oil) 5 days before they are killed. They are fasted 12 h before they are decapitated and allowed to bleed. The livers are aseptically removed and washed in cold 0.15 *M* KCl. All steps are performed at 0 to 4°C with cold and sterile solutions and glassware. The livers are minced with sterile scissors in three volumes of 0.15 *M* KCl (3 ml/g wet liver) and homogenized with a Potter-Elvehjem apparatus with a Teflon pestle. The omogenate is centrifuged for 10 min at 9000 x *g*, and the supernatant (S-9) is decanted and stored in convenient aliquots at -80°C. For S-9 from φB-induced rat livers, the same procedure as described above is followed except that the rats are given 0.1% sodium phenobarbital in drinking water for 1 week before they are killed.

MEDIA

Top agar (0.6% Difco-Bacto agar, 0.5% NaCl) is autoclaved and stored in 100-ml bottles at room temperature. Before use, the agar is melted (in an autoclave or in a steam bath), and 10 ml of a sterile solution of 0.05 *mM* L-histidine-HCl, 0.5 *mM* biotin is added to the 100 ml of molten agar and mixed thoroughly.

Complete medium (23.5 g BBL standard methods agar in 1 l of distilled H_2O) is autoclaved and dispensed into 100x15 mm plastic petri plates (30 ml/plate).

Vogel-Bonner[3] medium E with 2% glucose and 1.5% Bacto-Difco agar is used as the minimal medium for mutagenesis assays and is prepared as follows:

Vogel-Bonner Salts (50x)

Warm distilled water	670 ml
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaHNH_4PO_4 \cdot 4H_2O$)	175 g

The above salts are added to the warm water (45°C) in the specified order. Each salt is dissolved completely before the next is added. When the salts are all dissolved, the solution is cooled to room temperature. About 5 ml of chloroform is added to the solution and stored in a capped bottle at room temperature.

Dissolve 15 g of Difco-Bacto agar in 1 l of water by autoclaving. Cool to about 60 to 70°C and add 20 ml of 50x Vogel-Bonner salt solution and 50 ml of sterile 40% glucose solution. Mix thoroughly, and dispense into 100x15 mm plastic petri plates (30 ml/plate). Other minimal media would presumably also serve the purpose.

PREPARATION OF S-9 MIX (ACTIVATION SYSTEM)

The S-9 mix contains the materials shown in Table G-2.

TABLE G-2. COMPOSITION OF S-9 MIX

Component*	Stock preparation	Volume (μl) of stock	Final concentration of component in mix (μmol/ml)
1. NADP	0.1 *M*	40	4
2. Glucose-6-phosphate	0.1 *M*	5	5
3. Sodium phosphate buffer (pH 7.4)	0.2 *M*	500	100
4. $MgCl_2$	0.4 *M*	20	8
5. KCl	1.65 *M*	20	33
6. Homogenate	standard KCl 9000 x *g* supernatant	100	approx. 25 mg of fresh tissue equivalent

*Components 1 and 2 are prepared in sterile distilled water and filter-sterilized before use. Components 3-5 are prepared in distilled water, sterilized, and maintained at 4°C. Component 6 is prepared in 0.15 *M* KCl and stored at -80°C until used.

POSITIVE CONTROL COMPOUNDS

Any assay performed should have a control in which the solvent or diluent is employed to see its effect on the rate of spontaneous revertants. In addition to this control, a known directly acting mutagen and the one that requires metabolic activation should be used to show that the assay system is working.

The positive control compounds shown in Table G-3 could be used in these assays.

TABLE G-3. POSITIVE CONTROL COMPOUNDS

	Concentration (μg/plate)	Activation	Response of strain		
			TA1535, TA100	TA98	TA1537
Sodium azide	2.5	-	+	-*	-*
9-Aminoacridine	10.0	-	-	-	+
2-Anthramine	5.0	+	+	+	+

* Weak responses may be obtained.

MUTAGENESIS ASSAY BY THE PREINCUBATION METHOD

It may be difficult to detect biological effects with the complex environmental mixtures due to (1) toxicity of the complex mixture or (2) low concentrations of the biologically active components in the complex mixture. The first problem should be dealt with by assaying the complex mixture for general toxicity towards bacterial survival before the mutagenesis assay is performed. The second problem should be dealt with at the level of concentration and fractionation of the complex mixtures. The following protocol is recommended for general toxicity.

Only one strain, TA1537, is used to determine the general toxicity range. Overnight culture in nutrient broth is diluted to obtain about 10^3 cells/ml. To the tubes containing 2 ml standard top agar are added: 0.1 ml of the diluted culture of TA1537, various amounts of the test material (the recommended levels are: 1000, 500, 100, and 10 μl/tube), and 0.5 ml of phosphate buffer, pH 7.4 (for nonactivation) or 0.5 ml of S-9 mix (for activation). The contents are mixed and poured on the surface of a bacterial complete plate. After the agar is hardened, the plates are incubated at 37°C for 48 h. Survival is compared with a control plate containing solvent but no chemical. Once the toxicity is determined, five dose levels within the 50% or greater survival part of the curve are selected for actual mutagenesis assays.

PREINCUBATION ASSAY

Four tester strains (TA1535, TA1537, TA98 and TA100) described earlier are used in the assay, and each data point is done in duplicate. The assay is conducted as follows:

To the sterile 13x100 mm test tubes containing 0.5 ml of the S-9 mix placed in an ice bath, an aliquot of the test compound (or positive control mutagen or solvent or diluent) and 0.1 ml of an overnight bacterial culture are added. S-9 mix should be replaced with 0.067 *M* phosphate buffer (pH 7.4) in nonactivation tests. The contents are mixed and the tubes are incubated at 37°C in a shaker for 20 min. At the end of the incubation, 2 ml of molten top agar (kept at 45°C) are added per tube and the contents are gently mixed. The contents are then poured onto the surface of a Vogel-Bonner minimal glucose agar plate (appropriately labeled). After the agar has solidified, the plates are incubated at 37°C for 2 days and the *his*$^+$ revertants are recorded. Table G-4 shows the results for 2-aminoanthracene, sodium azide, and dimethylnitrosamine tested by the standard plate incorporation method and the preincubation method.

TABLE G-4. MUTAGENICITY OF 2-ANTHRAMINE, SODIUM AZIDE, AND DIMETHYLNITROSAMINE

Concentration of mutagen	Mutagenicity (revertants/plate) in strain					
	TA1535*		TA98		TA100*	
	Plate incorporation	Preincubation	Plate incorporation	Preincubation	Plate incorporation	Preincubation
2-Anthramine (µg)						
0			36	45		
0.01			66	78		
0.1			431	841		
1.0			1,994	2,104		
10.0			1,903	2,207		
Sodium azide (µg)						
0	22	15			123	113
1.0	300	274			600	1,344
2.5	566	505			865	1,902
5.0	867	765			1,065	2,372
10.0	1,643	1,100			1,275	2,746
20.0	2,063	1,456			1,336	3,015
50.0	2,260	1,675			1,362	3,161
Dimethylnitrosamine (µl)						
0	15	29			113	161
1.0	22	35			176	164
2.5	23	35			182	164
5.0	28	41			176	173
10.0	20	349			178	516
20.0	25	583			209	1,090
50.0	2	24			0	122

*Mean of six replicate runs.

REVERTANT CONFIRMATION

Randomly selected *Salmonella* revertants should be picked from plates showing mutagenicity and confirmed for histidine independence by restreaking on minimal plates containing no histidine.

REPEAT TESTS

The test on each sample should be repeated within 2 weeks following the initial evaluation to confirm the results. The positive results obtained in the initial evaluation with or without PCB-induced rat liver S-9 are to be confirmed in the repeat test. If the results are negative in the initial evaluation in the presence or absence of PCB-induced rat liver S-9, it is suggested that in the repeat tests φB-induced rat liver S-9 be included in addition to the PCB-induced rat liver S-9. (It should be noted here that the liver from Ar-induced rats is the most efficient for detecting different classes of carcinogens. The liver from φB-induced rats is more efficient for detection of 2-acetylaminofluorene and many other aromatic amines, but it is very inefficient for detection of certain PAHs.) If the repeat test results are positive in the presence of φB-induced rat liver S-9, they should be reconfirmed by testing the material in the presence of φB-induced rat liver S-9 only. If the repeat test results are negative, no further testing is necessary. Figure G-2 gives the general scheme for evaluating the test material in the preincubation assay for four *Salmonella* tester strains.

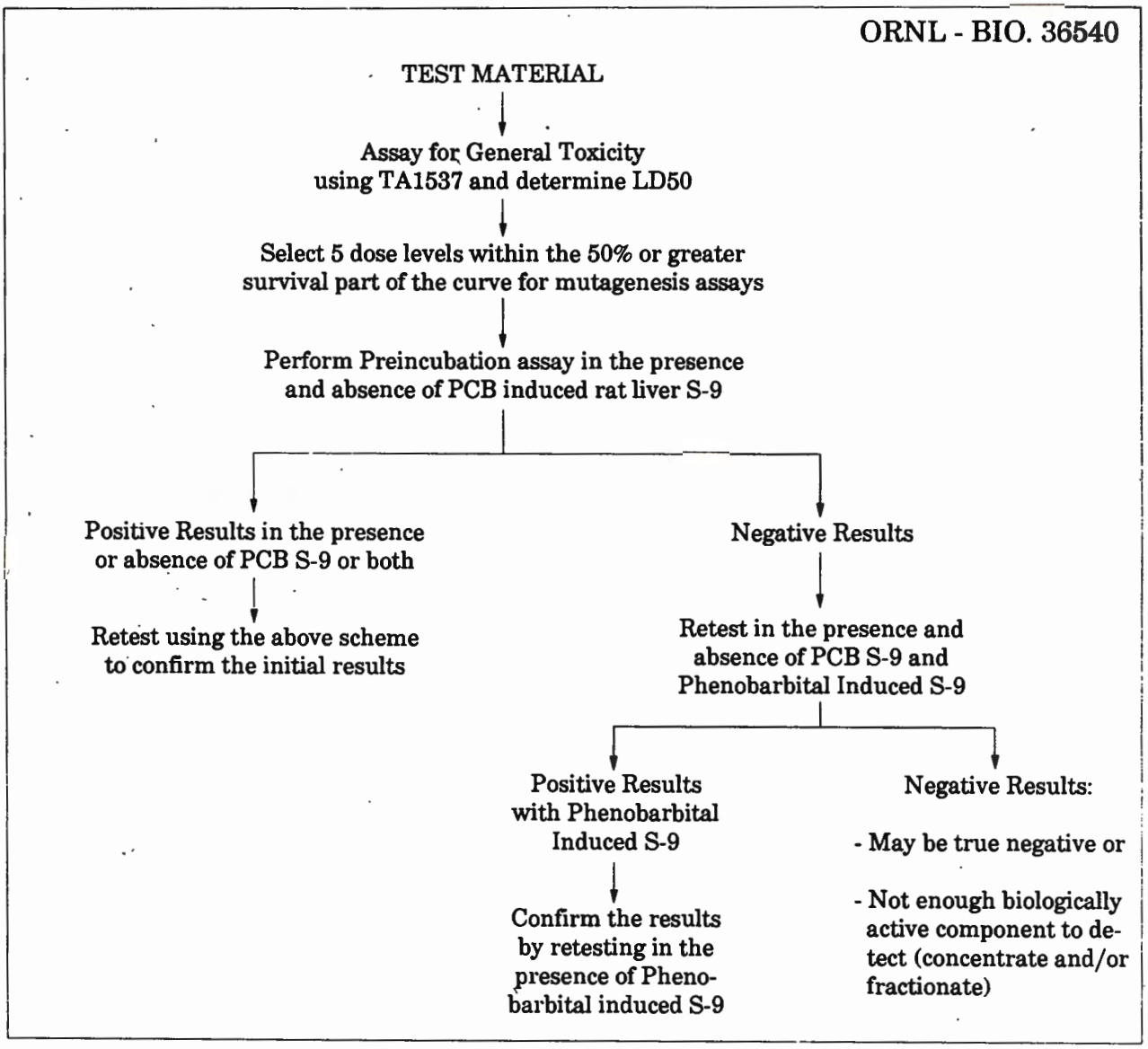

Figure G-2. General scheme.

REFERENCES FOR APPENDIX G

1. McCann,J.E., E. Choi, E. Yamasaki, and B.N. Ames. Detection of Carcinogens as Mutagens in the *Salmonella*/ Microsome Test: Assay of 300 Chemicals. *Proc. Natl. Acad. Sci. USA, 72:5135, 1975.*

2. Ames,B.N., J. McCann, and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian-Microsome Mutagenicity Test. *Mutat. Res., 31:347, 1975.*

3. Vogel,H.J., and D.M. Bonner. Acetylornithinase of *Escherichia coli*: Partial Purification and Some Properties. *J. Biol. Chem., 218:97, 1956.*

APPENDIX H

Saccharomyces cerevisiae GENE MUTATION ASSAY

Both forward and reverse mutation can be monitored in the haploid strain XL7-10B.[1] It has the genotype *αρ⁺ CAN1 his1-7 lysl-1 ural.*

FORWARD MUTATION TO CANAVANINE RESISTANCE (*CAN1-> can1*)

Canavanine is a toxic arginine analog to which yeast is normally sensitive. Resistence to canavanine has been shown to be almost exclusively due to mutational inactivation of the arginine permease. The permease gene (*CAN1*) has been estimated to be approximately 7700 nucleotides long, hence it offers a very large mutational 'target'. *CAN1* is mutated by both frameshift and base-pair substitution inducing mutagens; in addition, deletions and chromosomal rearrangements with breakpoints in *CAN1* should also be recoverable.

REVERSION OF *his1-7*

The *his1-7* mutation is a missense mutation resulting from a base-pair substitution in a histidine biosynthesis gene. This mutation confers a requirement for the amino acid histidine. Back mutation by base-pair substitution at the original mutant site removes the histidine requirement. Further, *his1-7* reverts by second site mutation - a second base-pair substitution at another site which 'corrects' the original amino acid replacement in the enzyme protein by a second compensatory replacement. Since the reversion event is not limited to a single site, a broader spectrum of base-pairs substitutions can be detected. Also, owing to different modes of DNA repair in yeast, *his1-7* is reverted by mutagens which have been classified in bacterial systems as acting via a frameshift mechanism.

Both *CAN1* and *his1-7* mutate readily, and the mutant are subject to a positive selection method. Additionally, this system will tolerate a wide variety of assay conditions (e.g., stationary phase versus log phase cells or presence or absence of a mammalian microsomal activation system) without requiring modification of the mutant selection procedure or affecting the recovery of mutants.

SUPPLIES AND EQUIPMENT

YPD, SC-ARG+CAN, and SC-HIS agar plates, prepoured
Sterile solution of 0.067 *M* K_2HPO_4
Sterile solution of 10% (w/v) $Na_2S_2O_3$ on ice
Clinical centrifuge and sterile centrifuge tubes
Sterile plastic test tubes with sealing caps (16x100 mm is convenient - available from Falcon)
Shaking water bath set at 30°C (rotary preferred)
A supply of sterile 10-, 5-, and 1-ml pipettes and tips for microliter pipetor
Sufficient S-9 mix[2] for activated assays (prepare fresh and hold on ice, maximum 3 h)
Ice bath for stopping assay
Sterile 0.067 *M* K_2HPO_4 dilution blanks (in plastic tubes as above)
Glass bacterial spreader and alcohol for flaming
Alcohol or gas burner
Protective gloves for handling test materials
Test material in aqueous or dimethylsulfoxide solution
Hemocytometer and compound microscope.

MEDIA

Media have the composition shown below and are sterilized by autoclaving.

YPD	
1% Difco yeast extract	6 g
2% Difco-Bacto-peptone	12 g
2% dextrose	12 g
2% Difco-Bacto-agar	12 g
distilled water	600 ml

(For broth leave out agar)

SD

0.67% Difco yeast nitrogen base without amino acid	4 g
2% dextrose	12 g
2% Difco-Bacto-agar	12 g
distilled water	600 ml

A modified synthetic complete is prepared by the following addition to SD (concentrations in mg/l).

SC

adenine sulfate	20
uracil	20
L-tryptophan	20
L-histidine HCl	20
L-arginine HCl	20
L-methionine	20
L-leucine	30
L-lysine HCl	30

SC-ARG+CAN is prepared by deleting arginine and adding filter-sterilized canavanine sulfate (40 mg/l) after autoclaving. SC-HIS is prepared by deleting histidine.

ASSAY METHODOLOGY

Suspend a well-formed isolated colony of the appropriate tester strain in 0.067 *M* K_2HPO_4 and determine the cell concentration using the hemocytometer. Prepare a dilution series and inoculate 25 ml of YPD broth with approximately 200 cells. Grow 2-3 days with vigorous shaking at 30°C until late stationary phase.

Centrifuge the stationary-phase culture and resuspend in buffer. Adjust cell concentration to $2x10^9$ cells/ml.

Place sufficient tubes for the assay in the ice bath. To each tube add: up to 0.5 ml aqueous test material (or up to 100 µl of dimethylsulfoxide solution), 0.4 ml of S-9 mix (for activated assays), and sufficient 0.067 *M* K_2HPO_4 to bring the volume in each tube to 0.9 ml. Finally, add 0.1 ml of the yeast suspension to each assay tube. Seal the caps.

Without delay, place the assay tubes in the 30°C shaking water bath. At least a 3-h and a 20-h incubation should be performed.

Stop the assay by placing the tubes in the ice bath and adding 1.0 ml of ice-cold 10% $Na_2S_2O_3$ to each tube.

PLATING

Plate the stopped incubation mixture directly on SC-ARG+CAN and SC-HIS (in duplicate, 0.1 ml/plate). Dilute the stopped mixture 10^{-5} and plate on YPD to determine survival.

Spread to dryness, flaming the spreader for each plate. Incubate YPD plates 3 days at 30°C, others 5 days. Count the plates. Calculate the percent survival and the mutation frequency based on surviving titer and note the mutation yield.

NOTES

Activation: φB-induced and Ar- (or substitute) induced S-9 are used, as per Ames.[2]

Enzyme titration: after the dose giving 50% survival, or the highest dose applicable (if the substance is nontoxic), is determined, the activation system is optimized by titration with varying amounts of S-9.

REFERENCES FOR APPENDIX H

1. Strain available from F.W. Larimer, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830.
2. Ames, B.N., J. McCann, and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. *Mutat. Res., 31:347, 1975.*

APPENDIX I

BACTERIAL DNA REPAIR ASSAY

DNA repair tests do not measure mutation per se, but DNA damage induced by chemical treatment of a cell. Microbial test systems measure this damage as cell killing. Test system employ paired, identical cells, except one has the normal DNA repair capabilities and one lacks a specific step (or steps) in the enzyme pathways responsible for DNA repair. Preferential killing of the repair-deficient strain by the test chemical implies that the chemical exerts its killing effects by reacting with the cells' DNA and, therefore, may be mutagenic. This implication may not be valid in all cases, since the test cannot separate a purely lethal

DNA effect from one that also has a mutagenic component.

The following protocol describes a generalized DNA repair assay which can utilize any of the major bacterial 'repair' strains, i.e., the *Bacillus subtilis* rec^+ - rec^- pair,[1] *Escherichia coli* $polA^+$ - $polA^-$,[2] or *Salmonella typhimurium* $uvrB^+$ - $uvrB^-$.[3] These systems are all based on the hypersensitivity of repair-defective bacteria to the lethal effects of DNA-modifying chemicals.

STRAIN MAINTENANCE

The source references for the strains chosen give details for the maintenance of master cultures. The repair phenotypes are conveniently verified by checking for UV sensitivity as follows:

The tester strains are parallel-streaked across individual nutrient agar plates and half of each plate is irradiated with a G.E. 15 W germicidal lamp at a distance of 33 cm. The duration of UV exposure is 6 s, after which the plates are incubated overnight at 37°C. The repair-deficient strain should show growth only on the *unirradiated* side of the plate, while the repair-proficient strain should show growth on both sides of the plate.

SUPPLIES AND EQUIPMENT

Prepoured nutrient agar plates
Sterile solution of 0.067 *M* K_2HPO_4
Sterile solution of 10% (w/v) $Na_2S_2O_3$ on ice
Clinical centrifuge and sterile centrifuge tubes
Sterile plastic test tubes with sealing caps (16x100 mm is convenient - available from Falcon)
Shaking water bath set at 37°C (rotary preferred)
A supply of sterile 10-, 5- and 1-ml pipettes and tips for microliter pipetor
Sufficient S-9 mix[3] for activated assays (prepare fresh and hold on ice, maximum 3 h)
Ice bath for stopping assay
Sterile 0.067 *M* K_2HPO_4 dilution blanks (in plastic tubes as above)
Glass bacterial spreader and alcohol for flaming
Alcohol or gas burner
Protective gloves for handling test materials
Test material in aqueous or dimethylsulfoxide solution.

MEDIA

Nutrient broth is composed of 8 g Difco-Bacto nutrient broth, 5 g NaCl, and distilled water to 1 l; sterilization is by autoclaving. Nutrient agar is nutrient broth solidified with 2% Difco-Bacto agar.

REPAIR ASSAY

Prepare overnight at 37°C nutrient broth cultures of each tester strain; store at 4°C.

0.1 ml of each bacterial culture will be required for each respective assay point. Centrifuge on adeguate volume of each culture, discard the broth supernatant, and resuspend the bacteria in a like volume of 0.067 *M* K_2HPO_4.

Place sufficient tubes for the assay in the ice bath. To each tube add: up to 0.5 ml of aqueous test material (or up to 50 µl of dimethylsulfoxide solution), 0.4 ml of S-9 mix (for activated assays), and sufficient 0.067 *M* K_2HPO_4 to bring the volume in each tube to 0.9 ml. Finally add 0.1 ml of the appropriate bacterial suspension to each assay tubes. Seal the caps.

Without delay, place the assay tubes in the 37°C shaking water bath. Incubate unactivated assays for 20 min, activated assays for 2 h.

Stop the assay by placing the tubes in the ice bath and adding 1.0 ml of ice-cold 10% $Na_2S_2O_3$ to each tube.

PLATING

Prepare the following serial dilutions from each stopped assay tube: 1:100, 1:10, 1:10, 1:10, using the 0.067 *M* K_2HPO_4 dilution blanks.

For each dilution, pipet 0.1 ml onto duplicate nutrient agar plates. Spread to dryness, flaming the spreader for each plate.

Incubate the plates inverted at 37°C overnight.

Count the plates. Calculate percent survival for each strain at each assay point, relative to untreated controls.

REFERENCES FOR APPENDIX I

1. Kada, T., K. Tutikawa, and Y. Sadaie. In vitro and Host-Mediated "Rec-Assay" Procedures for Screening Chemical Mutagens; and Phloxine, a Mutagenic Red Dye Detected.
Mutat. Res., 16:165, 1972.
2. Slater, E.E., M.D. Anderson, and H.S. Rosenkranz. Rapid Detection of Mutagens and Carcinogens.
Cancer Res., 31:970, 1971.
3. Ames, B.N., J. McCann, and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test.
Mutat. Res., 31:347, 1975.