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Identification and Evaluation of Potential Physiological Toxicity Assays

Final Report

IDENTIFICATION AND EVALUATION OF POTENTIAL PHYSIOLOGICAL TOXICITY ASSAYS

by

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SUMMARY

Battelle's Columbus Laboratories has contracted with the Office of Pesticides and Toxic Substances, U.S. Environmental Protection Agency, to develop a list of physiological assays as potential toxicity screening tests and to assess the strengths and weaknesses of these assays (Contract No. 68-01-5043). After an extensive literature search, Battelle has compiled a list of 24 assays, covering all of the categories cited by OPTS/EPA in its Technical Directive. Brief descriptions of assay methods and tables containing critiques of each assay are presented, along with literature references for all of the assays.

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INTRODUCTION

In this report, Battelle's Columbus Laboratories develops a list of potential cellular, organellar, and enzymatic toxicity tests (here collectively referred to as physiological toxicity tests or assays) for rapid screening of potential toxicants by the Office of Pesticides and Toxic Substances-Environmental Protection Agency (OPTS/EPA, Contract No. 68-01-5043). This study also documents, on the basis of a review of the published scientific literature and of ongoing research efforts, the strengths and weaknesses of selected physiological toxicity test methods.

At present, the testing strategies proposed for premanufacture evaluation of chemical hazards to ecological systems utilize 96- or 48-hour acute toxicity tests on fish or invertebrates, respectively, as the primary (or only) screening methods to identify the need for further testing. There are several problems with these assays. First, such acute tests are usually poor predictors of the results of chronic studies, phytotoxicity tests, behavioral tests, and multispecies ecological studies. Second, the standard short-term acute tests depend significantly, not only on the toxic biological activity of a chemical substance, but also on its solubility, dispersability, and/or ability to penetrate target cells or organs in the test organisms. Such penetration is highly variable among species. Third, the time and cost of the standard acute tests mentioned above are great.

To circumvent the problems arising from the fish and invertebrate toxicity tests, a battery of rapid, in vitro, physiological tests could be developed as a first screening tier of a step-sequenced testing strategy for assessing ecological effects of chemicals. These physiological assays would measure effects on major metabolic and cellular functions, and from these functions, would attempt to predict effects on various test species and on ecosystem function. It is further assumed by this approach that chronic effects are the result of chemical toxicity at the cellular or subcellular level, and that these rapid, physiological tests can predict chronic effects. In sum, physiological assays might best serve as preliminary tests in ecological effects test schemes.

Described below are the theory and experimental procedures of these potential physiological toxicity assays. Each assay method is evaluated by several criteria, including the advantages and limitations, response to chemicals, and special equipment required. Pertinent references are included along with the criteria for each assay, as well as in bibliographic form at the end of the report.

Initial Identification and Screening of Potential Assays

In the initial stage of Battelle's literature search (see Appendix), some 45 potential toxicity tests were identified for consideration by OPTS/EPA. Potential assays were either of particular interest to OPTS/EPA, referenced in Woodard (1976), or independently identified by Battelle.

Of the 45 physiological assays considered, 21 have little promise for development as rapid toxicity screening tests (Table 1). The assays marked with an asterik (*) in Table 1 were not considered strong candidates for rapid toxicity screens because they required use of isolated organs and tissues. Isolated organs and tissues have been very useful in studying the mechanisms of toxic action and in providing an understanding of mechanisms by which chemicals exert toxic effects. In this role, such systems should become increasingly important. However, the use of isolated organs has serious limitations for studying physiological or toxicological effects, partly because of the modulating systems existing in the whole organism which can either increase or decrease a chemical effect. The usefulness of organ systems for rapid toxicological screening remains limited, primarily because such systems are most useful for screening large numbers of chemicals for a site-specific effect as opposed to screening a chemical for multiple biological effects. Also, in most cases, isolated organ assay systems use almost as many animals as an in vivo test would require. Consequently, there are little savings in total animal usage. Given these considerations, no rapid, physiological toxicity assays using tissue or organs are critiqued in this report.

Other potential assays (oxidative activity in rabbit endothelium, chromosome breakage in human leukocytes, oxygen consumption by human leukocytes) were rejected because of GLP problems. Endothelia and leukocytes

TABLE 1. ASSAYS CONSIDERED BUT NOT EVALUATED IN DETAIL IN THIS REPORT

Plant callus growth
Inhibition of cell division of plant suspension cultures
Glycogen conversion in perfused rat liver*
Malting
Inhibition of axon myelation*
Invertase activity in duodenum culture*
Epithelial growth in mouse kidney tissue*
Cholinesterase inhibition in cerebral cortex*
Collagen synthesis in human pleura cultures*
Vitamin B_{12} uptake in monkey illeum*
Tracheal muco-ciliary transport rate*
Aldosterone synthesis in the adrenal gland*
Fatty acid synthesis in adipose tissue*
Mutagenesis in Saccharomyces**
Mitotic frequency in duck embryonic lung cells (L132) †
Oxidative activity in rabbit endothelium †
Chromosome breakage in human leukocytes †
Enzyme leakage in perfused liver*
Oxygen consumption by human leukocytes †
Osmotic and ionic changes in leaf guard cells
Inhibition of regeneration in Hydra

* Assays described in Woodard (1976)--Organs and Tissues.
** Assays described in Woodard (1976)--Bacteria, Fungi, Protozoa, and Plant Cells.
† Assays described in Woodard (1976)--Mammalian and Avian Cell Culture Systems.

are genetically and developmentally heterogeneous, and thus create problems with quality control and uniformity of response. Malting, osmotic and ionic changes in leaf guard cells and inhibition of regeneration in Hydra were not considered promising assays because a very small, if any, data base on toxic chemical effects existed. Those potential toxicity screens involving plant tissue cultures (plant callus growth, inhibition of cell division of plant suspension cultures) were rejected because a very long time (as much as 2 months) is required to complete a toxicity assay involving callus or suspension cultures. Also, plant cell cultures are easily contaminated, especially since aseptic conditions must be maintained without antibiotics for the duration of the assay. The final two rejected assays [mutagenesis in Saccharomyces and mitotic frequency in duck embryonic lung cells (L132)] may be effective for screening a wide variety of mutagens. But, while all mutagens are toxicants, not all toxicants are mutagens. These assays are probably responsive only to chemicals that interact with or influence DNA.

The remaining assays, which are evaluated in this report, appeared reproducible, well-documented, and straightforward (Table 2).

The selection of these final 24 assays was based on several criteria. First, the test organism in each of these assays is either commercially available or easily grown or cultured from commercially available materials (e.g., seeds) (Table 3). Second, the methods for each assay are well documented and have been performed in many laboratories worldwide. For example, estimates of RuDP carboxylase activity have been made in many laboratories throughout the U.S., Japan, Europe, and many other locations. Third, many of the physiological processes measured by these assays have been tested with some chemicals for possible toxic effects. So, at least some data base on toxic chemical effects is available for each of the 24 assays. Fourth, the selected assays are generally more rapid (assays per unit time) than the rejected candidate assays.

Each of these protocols is currently the most streamlined process available. With more research and development, the potential for further streamlining exists.

TABLE 2. POTENTIAL TOXICITY ASSAYS ADDRESSED IN THIS REPORT

Cellular process	Specific assay
Nitrogen fixation*	Acetylene reduction
Photosynthesis*	Hill reaction Greening RuDP carboxylase activity Photosynthetic oxygen evolution
Respiration*	Respiration in HeLa cells**
High-energy phosphate production	Adenylate energy charge
Growth and cell division*	Cloning L929 mouse cells** Protozoan clonal viability Human KB cell growth rate Human embryonic lung fibroblast (WI-38) cytotoxicity Mitogen stimulation of lymphocytes Chick embryo development** Trypan blue dye exclusion by human KB cells
Catalysis (enzymatic activities)	RNA polymerase activity Adenyl cyclase activity Lysosomal enzyme release Macromolecular synthesis in KB cells
Other cellular processes	Cyclosis* Hemolysis* Protozoan vacuole contraction* Protozoan motility* Phagocytosis by alveolar macrophages Amino acid transport

*Subjects mentioned in EPA directive to Battelle.
**Subjects mentioned in Woodard (1976).

TABLE 3. COMMERCIAL SOURCES OF TEST ORGANISMS

Organism	Source
Algae:	
Scenedesmus	ATCC*
Euglena	ATCC
Chlorella	ATCC
Chlamydomonas	ATCC
Chara	Starr**
Elodea	Starr
Nitella	Starr
Bacteria:	
Azotobacter vinelandii	ATCC
Escherichia coli	ATCC
Clostridium pasteurianum	ATCC
Protozoan:	
Tetrahymena pyriformis	ATCC
Human Cell Lines	
HeLa	ATCC
KB	ATCC
WI-38	ATCC
Human erythrocytes	Calbiochem
Other mammalian Cell Lines:	
Mouse L929	ATCC
Mouse lymphocytes	Mouse colony
Rabbit alveolar macrophages	Rabbit colony
Rat erythrocytes	Rat colony
Higher Plant (seeds):	
Spinach oleracea	DeKalb Agresearch †
Hordeum vulgare	DeKalb Agresearch
Phaseolus vulgaris	DeKalb Agresearch
Canavalia ensiformis	DeKalb Agresearch
Higher Animal:	
Mice	Jackson Labs, Bar Harbor, ME
Rabbits	Jackson Labs, Bar Harbor, ME
Rats	Jackson Labs, Bar Harbor, ME
Chickens	Reliable local hatchery

*ATCC--American Type Culture Collection.

**Dr. Richard Starr, algal culture collection, University of Texas at Austin.

† Or other company with genetically homogeneous seeds [good sources can be confirmed by the American Seed Trade Association (ASTA)].

Organization of Information About Assays

In the following pages, 24 physiological assays which could be developed for rapid toxicity testing are described and assessed. As shown in Table 2, assays are organized under seven different cellular processes which were suggested by OPTS/EPA.

The information on each assay is divided into two parts. First, there is a brief description of each assay along with the biological meaning of the test results. Second, data tables accompany each assay description for easy access of relevant information. The protocols and data tables are complementary, so both should be considered for objective evaluation of the individual assays. The information organization described above allows easy evaluation and comparison of individual assays.

A tabulation summarizing pertinent data on the assays is presented in the Discussion and Recommendations section at the beginning of the report.

Explanation of Data Tables Accompanying Each Assay

Each potential toxicity assay has been assessed according to seven criteria. The scope and meaning of each criterion are given below.

Test Organism--

A representative test organism (or organisms) is suggested for each assay. Each organism mentioned in this report has been the object of most of the particular studies in toxicity evaluations. Observations have been made on these organisms, and on others mentioned in certain assays, in terms of growth and survival, hallmark metabolic process, or changes in gross morphology or ultramorphology. These test organisms were also selected because relatively large quantities of these cells or tissues can be quickly grown or inexpensively purchased (Table 3). Enzymes used in certain toxicity assays (e.g., RNA polymerase activity) were selected because of their abundance in particular tissues, commercial availability, stability of activity, or relevance of the metabolic process in which the enzyme participates.

Advantages and Limitations--

Some advantages and limitations of each method are listed in the following tables and these are straightforward in interpretation.

One factor considered was the level of technical skill needed to perform each assay. We identified three different levels of technical competency. Highly skilled technicians are defined as individuals with master's degrees and research experience. Skilled technicians are individuals who have had college or university research experience and who hold a technical bachelor's degree. Unskilled technicians are defined as individuals with a minimal scientific background (e.g., associate or junior college degree). If unskilled technicians could be used to perform an assay, this fact was listed as an advantage. On the other hand, if a skilled or highly skilled technician was required, this fact was considered a limitation. As each particular assay method is standardized and becomes routine for a given laboratory, a lesser degree of technical skill than mentioned in the table for that assay may be utilized.

Response to Chemicals--

Each potential toxicity assay was assessed with regard to its response to certain chemicals. Most of these assays have not been developed as toxicity tests as such, but were used to study certain physiological processes. Any toxicity testing has been incidental (i.e., to determine physiological effect of a chemical, not to determine a chemical's toxicity).

A sampling of chemicals and chemical classes has been included in each table to give an idea about the range of chemicals that affect the assay. Other chemicals may (or may not) affect the physiological process in each assay, but these chemicals have either not been studied or were not revealed during our literature search.

In every assay listed (except hemolysis and lysosomal enzyme release), the term "response to chemicals" refers to chemical inhibition of the observed physiological process. For instance, in respiration in HeLa cells, malonate lowers (or in sufficiently high concentrations, stops) mitochondrial oxygen uptake. The chemicals listed in hemolysis, however, promote the lysis

of red blood cells rather than inhibit it. In each assay the chemicals listed affect the physiological process in a concentration-dependent manner.

When available, the concentrations of some chemicals affecting the assays were included. Affecting chemical concentrations were abbreviated EC, I, LC, or MEC. EC_x (effective concentration) is the concentration that induces detrimental effects in x percent of the test organisms. For example, in the hemolysis assay, EC_{50} would be a chemical concentration that causes 50 percent of the red blood cells to lyse. I_x is defined as the chemical concentration causing x percent inhibition of a physiological process (e.g., greening or enzymatic catalysis activity). LC_x (lethal concentration) is the chemical concentration causing the death of x percent of the test cells or organisms. For instance, LC_x is used for chemicals causing chick embryo death. MEC (minimal effective concentration) is defined as the lowest chemical concentration at which toxic effects are first observed. MEC is used in the mammalian cell culture assays.

In the assays examined in this report (Table 2), the test chemicals have elicited a unidirectional physiological response (e.g., inhibition of respiration, inhibition of KB cell growth rate). It is possible that future studies may reveal chemicals having an opposite effect (e.g., stimulation of respiration, stimulation of KB cell growth rate). Would a chemical that stimulated, instead of inhibited, a cellular process be considered toxic?

Assay Time--

The times required for each assay (including preparation time, technician time, etc.) were assessed and are included in each data table. There are four numbers listed opposite Assay Time in each table. The first number is the time (in hours) to perform an assay set (i.e., one chemical, three replicates of each of five concentrations). If several replicates of different chemicals could be assayed simultaneously, this was considered an advantage. It was considered a limitation when an assay set required long periods of time or when only one individual assay could be completed at a time. The second number listed in the Assay Time category is the total time for an assay set to be completed. This includes cell growth period, solution preparation, data recording, and laboratory cleanup and is an estimate of time from

start-up to expression of results. The third number is total technician time. Since technicians can do several operations simultaneously (e.g., prepare solutions during cell growth period) and some processes may continue unsupervised overnight, this number may be significantly less than the total assay time. The last number is the administrative time required. This includes Ph.D. supervision time, managerial time, data analysis, and reporting of results. It was necessary to separate this from technician time since a more highly trained person is usually required for administration. These times are estimated for assays that are in the late developmental stage. As the assay method is standardized and becomes routine, times would probably become shorter.

Specialized Equipment--

Specialized equipment needed to accomplish each assay is listed in the table accompanying each assay description. Not all equipment required for each test is presented. All of these potential toxicity assays can be performed routinely only in a laboratory equipped with basic analytical instruments (centrifuges, balances, etc.), minimal cell culture equipment (incubators, culture dishes or flasks, etc.), and biochemicals (buffers, metabolites, etc.). If the other necessary special equipment was rare or costly, this was considered a limitation. For example, mammalian cell culture facilities, needed in many of the mammalian cell cytotoxicity assays, require a sterile working area such as laminar flow hood or transfer room. Such apparatus is probably not standard in most laboratories and may cost as much as $7000. Other special equipment, such as a spectrophotometer or colorimeter, is relatively inexpensive and is found in many laboratories. Such special equipment is listed in the data tables but is not considered a limitation.

The assays listed in Table 2 already involve certain levels of automation. For example, protozoan motility utilizes a microphotography unit and RNA polymerase activity uses a multipurpose filtration manifold. Many other assays (e.g., mitogen stimulation of lymphocytes, adenyl cyclase activity, amino acid transport) are partially automated by using scintillation counters with statistical data analyzers. The need for automation of any particular assay is dependent on the volume of chemicals to be tested--if many chemicals

are to be tested, automation or development of automation would ensue. In several assay descriptions we suggest potential points for automation (e.g., use of computer and TV in protozoan motility assay).

Cost--

The estimated cost for an assay set is included in each data table as well as in a comprehensive comparison table at the end of the report (Table 29).

To calculate the estimated costs, the technician's hourly wage was multiplied by the total number of technician hours, and this was multiplied by a factor of 2.64. This is the estimated labor factor for Battelle's Bioenvironmental Sciences Section, and is used for determining the approximate total cost for performing a task, including labor, supplies, use of equipment, and use of other facilities (e.g., electricity, water, maintenance).

The technicians' hourly wages are based upon average pay for similar technicians at Battelle, including 2 weeks annual vacation and other fringe benefits. Annual salaries of these technicians are: highly skilled technician, $18,000; skilled technician $12,000; and unskilled technician, $9,500. The cost of supervision by a Ph.D. level research scientist and managerial costs are also included. These annual salaries are estimated to be $25,000 and $35,000 for a Ph.D. scientist and manager, respectively.

The approximate costs listed in this report are only for purposes of comparison of assays. Actual costs may vary 20 to 25 percent from these figures at different laboratories. Developmental work on the assays would be considerably higher than these estimates for semiroutine testing. As the test comes into routine use, however, costs could decline sharply because of simplified and standardized methods (disregarding inflation).

Data generated in any one of the assays described in this report would be analyzed by routine statistical methods (e.g., variance analysis).

Assays from Woodard (1976)

In the report by Woodard (1976) to OPTS/EPA, potential physiological toxicity assays for studies on chemicals were reviewed under four categories:

(1) use of fertilized eggs, (2) use of isolated organs and tissues, (3) use of mammalian and avian cell cultures, and (4) use of bacteria, fungi, protozoa, and plant cells. In the following paragraphs, we consider in vitro tests derived from each of Woodard's test groupings.

The literature on the use of fertilized eggs in studies on chemicals focuses almost exclusively on the development and use of fertilized chicken eggs as a toxicity bioassay. The production of abnormalities in the developing embryo as a result of the administration of thallium was first demonstrated by Karnofsky in 1950 using the fertile chicken egg. However, the chick embryo development assay requires a long period of time to complete (1 month), calls for expensive specialized equipment, and does not have a universally standardized end point such as embryo death or abnormal limb development (Table 16). In general, the use of fertilized chicken eggs has enjoyed some attention in studies on the teratogenic potential of chemicals, but this method is still regarded by toxicologists as only marginally useful *in screening for other types of toxic effects.*

Toxicity tests have also been conducted using two types of invertebrate eggs as test subjects. However, few data exist concerning chemical effects on hatching of brine shrimp or on the early development of sea urchin embryos, and a tremendous amount of developmental research would be needed to adapt these assays for routine toxicity testing. As for present data on these two test systems, the inhibitory or stimulatory effects on hatching or development apparently do not correlate with the carcinogenic effects of known chemical compounds tested (Woodard, 1976). So these assays are apparently not immediately useful as potential toxicity screens.

Woodard's category on the use of isolated organs and tissues in studies on chemicals was reviewed. The advantages and limitations of these bioassay systems are discussed on page 2 in relationship to Table 1.

Several rapid, potential toxicity assays using mammalian cell culture, bacterial, protozoan, and plant test systems are critiqued later in this report.

Cytotoxicity assays employing mammalian cells in culture measure quantitatively cellular and metabolic impairment or death resulting from in vitro exposure to soluble and particulate toxicants. Mammalian cells derived

from various tissues and organs can be maintained as short-term primary cultures or, in some cases, as continuous cell strains or lines. Primary cultures exhibit many of the metabolic and functional attributes of the original tissue. Some of these attributes may be lost after a prolonged time in culture.

There are certain requirements basic to any assay that requires the use of mammalian cells in vitro. Paramount among these are aseptic facilities for the propagation and handling of cultured cells and qualified personnel trained in safe and proper cell culture technique.

There are many advantages in using mammalian cell culture systems in toxicity assays. First, they are generally more rapid and less costly than whole animal tests. Second, a lesser quantity of potential toxicant is required for these in vitro tests. Third, specific physiological or biochemical alterations are more easily evaluated in cell culture systems, and fourth, the systems provide useful information about the relative cellular toxicity of unknown samples (Woodard, 1976).

Cell culture toxicity screens also have several drawbacks. Since the assays employ isolated cells and not intact animals, they can provide only preliminary information about the ultimate health hazards of toxic chemicals. In many instances, some metabolic action in an animal renders a chemical toxic or nontoxic. So, a chemical which appears toxic at the cellular level may actually be innocuous at the tissue or higher level because of metabolic deactivation. Likewise, a toxic chemical could appear nontoxic at the cellular level since metabolic activation of a chemical to a toxic form could occur in vivo but might not occur in cell culture.

Another disadvantage is that cell culture test systems may become contaminated with latent viruses or Mycoplasma sp., which can alter cellular metabolism. Also, media constituents (such as calf serum) must be carefully monitored and controlled since they may affect cellular metabolism or form complexes with the test chemical (Woodard, 1976).

Both neoplastic (tumor-derived) and nonneoplastic (primary) cell lines are utilized in assays described in this report. Although neoplastic cells are abnormal and have probably lost some metabolic capabilities as compared with primary cultures, they respond equally well in many cytotoxicity

assays. Neoplastic cells are generally used because they grow rapidly and are more readily propagated than primary cultures. The only neoplastic cell lines used include human HeLa and KB. Nonneoplastic cell lines utilized include human WI-38, rabbit alveolar macrophages, mouse L929, and mouse lymphocytes. Other mammalian cell types can be used as alternatives to these cell lines.

Several of the cell culture assays described here could be combined to form one assay which could assess several parameters. This would provide a more cost-effective means for using cell culture systems for screening toxic chemicals.

As described by Woodard (1976), the use of nonmammalian cell systems in toxicity testing is now well established. The potential for bacteria, protozoan, and plant systems in physiological assays is also great. Most assays involving these systems are more rapid and less expensive than mammalian systems. Also, many potential toxicants can generally be screened simultaneously, and often only unskilled technicians are required to perform the test.

A drawback to the use of plant, bacteria, and protozoan systems in toxicity screens is the questionable extrapolation of data obtained from these systems to mammals. The converse is also true in that mammalian systems as toxicity screens cannot always be extrapolated to plant or microbial systems. Although all cells have certain structural and metabolic properties in common, certain processes which only occur in whole animals or plants (e.g., uptake and transportation of potential toxicants) still are not fully understood.

DISCUSSION AND RECOMMENDATIONS

The objective of this report was to identify potential physiological toxicity tests in the literature and to assess each on the basis of several criteria. Even though each assay has advantages and disadvantages, it is difficult to rank them on the basis of a literature review alone. Before any final decision on the utility of any assay is made, laboratory evaluation is necessary. However, on the basis of the literature review, it is possible to approximate the degree of laboratory development needed to adapt and validate these protocols as routine toxicity screens.

We have identified three levels of assay development: those requiring minimal development, those requiring some development, and those requiring significant development. Assays are placed in one of these categories on the basis of the criteria listed in Tables 28 through 32.

Assays that would require little development for use as toxicity assays (i.e., immediate validation) include greening, hemolysis, human KB cell growth rate, phagocytosis by alveolar macrophages, macromolecular synthesis in human KB cells, RNA polymerase activity, and human embryonic lung fibroblast (WI-38) cytotoxicity. Assays that would require some development for use as toxicity assays include acetylene reduction, the Hill reaction, RuDP carboxylase activity, adenylate energy charge, chick embryo development, protozoan clonal viability, cloning L929 mouse cells, trypan blue dye exclusion by human KB cells, protozoan motility, and amino acid transport. Assays that would require extensive development include cyclosis, protozoan vacuole contraction, photosynthetic oxygen evolution, respiration in HeLa cells, mitogen stimulation of lymphocytes, lysosomal enzyme release, and adenyl cyclase activity.

Some assays naturally drop from consideration. These tests meet few (or none) of the criteria used for assay evaluation (simplicity, rapidity, cost effectiveness, documentation, reproducibility, etc.). For example, cyclosis is one of the most expensive and time-consuming assays. It has a poor data base, and results are probably not ecologically significant. Protozoan vacuole contraction, photosynthetic oxygen evolution, and respiration in HeLa cells require extensive development and have been rejected for immediate use because several of the criteria are not optimum. Adenyl cyclase

activity, mitogen stimulation of lymphocytes, and lysosomal enzyme release have poor data bases with regard to toxic chemical effects. In addition, it appears difficult to relate results from adenyl cyclase activity to cellular or tissue toxicity. Mitogen stimulation of lymphocytes requires maintenance of an expensive mouse colony.

Those assays in the second category (some development) are more difficult to evaluate since their advantages and limitations are more equally balanced. In some assays (Hill reaction, chick embryo development) very good data bases on toxic chemical effects exist. However, there are GLP problems with the Hill reaction since chloroplast activity may vary. Chick embryo development requires a long time to complete and lacks a standardized end point. RuDP carboxylase activity has a poor data base and GLP problems, even though it is rapid and inexpensive. Protozoan motility is time consuming and expensive, but automation could make test results easier to obtain. The clonal assays (cloning L929 mouse cells and protozoan clonal viability) both have good data bases and are simple. However, each requires a long time to complete.

The membrane assays (amino acid transport, trypan blue dye exclusion) were not considered easily developed assays because they have poor data bases. Development of these assays should be considered because tests results can be extrapolated to all membranes. Even though acetylene reduction monitors a vital physiological and ecological process, the current assay method needs streamlining (e.g., use of a multisample gas chromatograph).

The remaining assays are rapid, simple, reproducible, cost-effective, and well documented. In many cases, it is advisable to combine several tests (or give a single test multiple end points) and to correlate the results to give the responses to chemicals broader ecological or biological meaning. Greening, Hill reaction, chlorophyll fluorescence, and a growth test (e.g., seedling growth) could possibly be combined to give a good indication of phytotoxicity if the same organism were used in all assays (Kratky and Warren, 1971).

It also would be possible to combine RNA polymerase activity and macromolecular synthesis in human KB cells to detect chemical inhibition of RNA synthesis. Phagocytosis by alveolar macrophages and amino acid transport

(and/or trypan blue dye exclusion by human KB cells) could be combined to detect inhibition of mammalian membrane function.

Hemolysis appears to be one of the best potential toxicity assays because it is cost-effective and can be performed by unskilled technicians. Also, the lysis of erythrocytes is a generally accepted standard of toxicity because mammalian tissues depend on hemoglobin for transport of gases and nutrients. Human KB cell growth rate and human embryonic lung fibroblast (WI-38) cytotoxicity also should require little development since they are already used as toxicity assays by the National Cancer Institute. Since these assays are well documented, simple, and inexpensive, they could probably be quickly validated as toxicity screens, with implementation following.

Comments

In evaluating and ranking these 24 potential physiological toxicity test methods, there are several points that merit consideration.

Physiological test methodologies have proved very useful for studying mechanisms of toxic action and for evaluating large numbers of toxic chemicals. Physiological tests (predominately in vitro) have several advantages over in vivo methods (e.g., time, cost, and quantitation of results), but results from physiological tests can at best give preliminary information on a chemical's toxicity. As described on page 13, an in vivo system may mediate a chemical's toxic activity by metabolic activation or deactivation. This cannot occur in in vitro systems. Hence, physiological methodologies could give false positive or negative results.

Results obtained from cellular, organellar, or enzymatic test systems cannot usually be extrapolated to ecosystem effects for several reasons. First, only one physiological parameter of a single test organism is monitored in each assay. These assays are by no means an intensified ecological study, and no direct extrapolations from these tests could effectively be made to ecosystem effects. Second, test organisms such as mammalian cells or algae are genetically homogeneous because these test cells are clonally derived. Since cells and tissue systems differ greatly in whole animals, these cellular systems are usually not good indicators of in vivo responses. Third, a

selected test organism may be unusually sensitive or insensitive to certain chemicals and give inconclusive (or false) results. Physiological test systems, both mammalian and nonmammalian, could be effectively used as toxicity screens to identify the need (or lack of need) for further testing.

Information obtained from one cellular physiological test can often be extrapolated to a more complex multicellular system because of certain structural and functional similarities. All cells are enclosed by virtually identical semipermeable membranes, contain DNA, and respire. It is not usually safe to extrapolate beyond this, however.

In summary, since most previous toxicity test methods have been in vivo, the effectiveness of cellular or subcellular test methods has yet to be demonstrated. The in vitro test methods have several advantages over in vivo ones (e.g., time and cost), but they still have certain practical and scientific limitations involving correlation of toxic effects on cellular metabolism to toxic effects in ecological systems.

POTENTIAL TOXICITY ASSAYS--DESCRIPTIONS, METHODS, AND ASSESSMENTS

NITROGEN FIXATION

Acetylene Reduction

The conversion of atmospheric nitrogen into organic compounds by living organisms is called nitrogen fixation. This process is carried out by microorganisms, including the free-living bacteria, blue-green algae, and bacteria associated in a symbiotic condition with plant roots. The enzyme nitrogenase catalyzes the transfer of electrons from an electron source to nitrogen, resulting finally in the production of ammonium ions. The acetylene-ethylene assay for nitrogen fixation is based on the nitrogenase-catalyzed reduction of acetylene to ethylene. Ethylene concentration is determined by using a gas chromatograph equipped with a hydrogen-flame analyzer.

This assay involves incubation of bacteria with an energy source and reductant in a flask sealed with a serum cap. After repeated flushing with a source of acetylene, the bacteria are added aseptically through the cap. The reaction mixture is incubated on a rotary shaker at 30 C for 30 minutes, and the incubation is stopped by addition of 0.5 ml 6N sulfuric acid. Samples of the gas phase are then measured with a hydrogen$_2$-flame ionization detector after gas chromatographic separation.

As described by Hardy et al (1968), the complete assay system contains 4 ml liquid volume and 36 ml gas volume. The liquid reaction mixture includes 50 mM Tris-HCl, 56 mM creatine phosphate, 5 mM ATP, 5 mM magnesium chloride, 20 mM disodium thiosulfate, 0.2 mg of creatine kinase, and 4 mg of heated extract of ammonia-grown A. vinelandii. The gas phase of the reaction mixture contains 0.1 atmosphere of acetylene and 0.9 atmosphere of helium. Chemicals to be tested are added to the reaction vessels at various concentrations. Inhibition of acetylene reduction, expressed as a percentage of control values, can be calculated for the various levels of a test chemical.

Monitoring the effects of toxic chemicals on nitrogen fixation is important because captured atmospheric nitrogen is converted into amino acids, the building blocks of all proteins.

Details on acetylene reduction are summarized in Table 4.

PHOTOSYNTHESIS

Hill Reaction

Robin Hill discovered that light-induced oxygen evolution can be observed in cell-free granular preparations (chloroplasts) extracted from green leaves. Illumination of such chloroplast preparations in the presence of artificial electron acceptors, such as ferricyanide or reducible dyes, causes evolution of oxygen and simultaneous reduction of the electron acceptor, according to the general equation

$$H_2O + A \xrightarrow{h\nu} AH_2 + 1/2\ O_2$$

where A is the electron acceptor and AH_2 is its reduced form. In a photosynthesizing plant, A is nicotinamide adenine dinucleotide phosphate (NADP). NADP accepts the electrons, and the reduced form of NADP is used to reduce carbon dioxide into sugars. However, in the in vitro Hill assay described below, dyes are used to accept the electrons liberated from water. As the dyes are reduced, they change color and this color change is quantitated.

For the Hill assay, chloroplasts are isolated from plants grown under controlled conditions or from batch cultures of Euglena or Chlorella. According to the chloroplast isolation method of Wald et al (1966), algae or leaves are homogenized with 0.5 M sucrose solution at 0 C for 30 seconds in a Waring Blendor. The suspension is then filtered through two layers of cheese cloth. The filtrate is centrifuged at 50 g for 10 minutes. The supernatant is then decanted and centrifuged for 10 minutes at 600 g. The supernatant is decanted and discarded. The pellet at the bottom, containing the chloroplasts, is suspended in 0.5 M sucrose. It is important to keep the chloroplasts at 0 C because they deteriorate rapidly at higher temperatures.

It is advisable to examine the chloroplast preparation under a microscope to ensure that the chloroplasts are of uniform size, intact, and free of other cellular debris.

TABLE 4. ACETYLENE REDUCTION

Criteria	Critique/Comments
Test Organisms	Either Azotobacter vinelandii or Clostridium pasteurianum may be used.
Advantages	The analytical method can detect as little as 1 picomole of ethylene. The tests organisms are simple to culture. The assay may be utilized in either the field or laboratory. The phase of the potential toxicant may be solid, liquid, or gas. A short time is required to obtain the results of this assay.
Limitations	Acetylene is a very explosive gas and requires care in handling. Nonnitrogenase catalysis of the reduction may occur. Specialized equipment is needed. The assay and gas chromatography must be performed by skilled technicians.
Response to Chemicals	Chlorinated aliphatics [trichloroacetic acid, ethylene glycol bis(trichloroacetate)] Arsenicals (cacodylic acid, disodium methanearsonate) Metabolic inhibitors (2,4-dinitrophenol) Gases (carbon monoxide-I_{100} is 0.18 atm)
Assay Time, hours	2.5*, 54**, 12†, 8†
Special Equipment	Gas chromatograph with a hydrogen-flame analyzer
Cost §	$620
References	Hardy et al, 1968 Rubinstein, et al, 1975

*Time for one assay set--three replicates of each of five concentrations of ore chemical.
**Total assay set time, including cell growth, solution preparation, and data recording.
† Total technician time, including GLP, performing assay, and solution preparation.
† Administrative time (Management, Ph.D. supervision, data analysis, and reporting).
§ Estimated cost for comparative purposes. Actual costs may differ 20 to 25 percent (see p. 11).

The Hill reaction assay mixture contains 2 ml of 0.1 M phosphate buffer (pH 6.5), 2 ml of 2.5 x 10^{-4} M dichlorophenolindophenol, 0.1 of chloroplast suspension, 1 ml of toxicant or chemical to be tested, and 5 ml of distilled water. Chemicals to be tested are incubated with the chloroplast suspension prior to addition of the electron acceptor. Each reaction mixture is then exposed to the same bright light for 10 minutes. Over the course of the 10 minutes, the absorbance of the dye is monitored at 620 nm (Wald et al, 1966). The effect of a potential toxicant on the rate of this photosynthetic reaction is reflected by the rate at which the dye is reduced and turns from blue to clear.

Details on the Hill reaction are summarized in Table 5.

Greening

Potential toxicants alter plants' chlorophyll content by a number of mechanisms. Chlorophyll biosynthesis is affected by specific chemical stimulation or inhibition of DNA, RNA, or protein synthesis. Some chemicals affect chloroplast development or structure, resulting in an altered chlorophyll content. Other chemicals degrade or induce the degradation of the chlorophyll molecule (Wolf, 1977).

To measure the effect of a chemical on chlorophyll accumulation, dark-grown plants are subjected to a series of chemical concentrations prior to greening. In detail, etiolated barley plants 7 to 9 days old are sprayed (misted) with solutions of a chemical. The spray is directed at the coleoptiles (or hypocotyl hooks) from above. For every 200 seedlings, about 50 ml of solution is used. Alternatively, seedlings could be grown in soil amended with toxicant. The seedlings are transferred to an irradiation chamber 1 hour after being treated. The plants are irradiated for 24 hours by white fluorescent lamps at an intensity of 1000 ftc. Except during white-light irradiation, plant material is handled in dim green light (Margulies, 1962).

Chlorophyll is extracted from 2-g leaf samples by heating in boiling water for 30 seconds, and then by grinding in a Virtis-type homogenizer with 80 percent acetone. The macerate is centrifuged, and the chlorophyll content of the resulting supernatant is measured spectrophotometrically at 663, 645,

TABLE 5. HILL REACTION

Criteria	Critique/Comments
Test Organisms	Spinach (Spinacia oleracea), Chlorella, Euglena
Advantages	The test organisms are simple to grow or are readily available. A short time is required to obtain the results of this assay. The assay is capable of detecting very minute quantities of potential toxicants. The assay can be performed by unskilled technicians.
Limitations	Chloroplast activity varies among preparations and declines with age. Reducing or oxidizing agents may interfere with and produce variation in the assay.
Response to Chemicals	Antibiotics (chloramphenicol-I_{90} is 4 mg/ml) Ureas (3-cyclooctyl-1,1-dimethylurea, 1-(2-methylcyclohexyl)-3-phenylurea) Herbicides (2-chloro-4,6-bis(isopropylamino)-s-triasine, 2-methoxy-4,6-bis(ethylamino)-s-triazine) Inorganic salts (ammonium chloride) Inorganic ions-heavy metals (cadmium, zinc)
Assay Time,* hours	3, 57, 18, 11
Special Equipment	Spectrophotometer (Beckman Spec 20) or colorimeter
Cost*	$820
References	Hill, 1937 Margulies, 1962 Moreland and Hill, 1962 Anderson and Boardman, 1964 Wald et al, 1966 Brown and Haselkorn, 1972 Hamp et al, 1975 Rubinstein et al, 1975

*See time and cost explanation, pp. 9-11 in text.

and 626 nm by the method of Anderson and Boardman (1964). This method takes into account the absorbance of protochlorophyll, the precursor of chlorophyll, as well as chlorophylls a and b. The following equations can then be solved to give the individual pigment concentrations in µg/ml:

$$C_a = 12.67E_{663} - 2.65E_{645} - 0.29E_{626}$$

$$C_b = -4.23E_{663} + 23.60E_{645} - 0.33E_{626}$$

$$P = -3.99E_{663} - 6.76E_{645} + 29.60E_{626}\ .$$

This bioassay is especially sensitive to photosynthetic and respiratory inhibitors, but results do not usually correlate with results from growth assays (Kratky and Warren, 1971).

Details on the greening assay are summarized in Table 6.

RuDP Carboxylase Activity

Ribulose-1,5-diphosphate carboxylase (RuDPCase) is a soluble enzyme localized in the chloroplast stroma of vascular plants. This enzyme catalyzes the primary fixation of carbon dioxide during photosynthesis in some monocots and in most dicots.

This assay employs cell-free extracts of leaves or algae. Leaves are obtained from spinach plants grown under controlled conditions and the algae Euglena and Chlamydomonas are easily grown in batch cultures. As described in the greening assay, test organisms are exposed to a chemical prior to isolation and determination of RuDPCase. Alternatively, chemicals to be tested can be incubated with the enzyme preparation prior to additon of the other reaction reagents (see below).

To prepare an extract, as described by Goldthwaite and Bogorad (1971), 1 g of leaves or algae is ground in a small Waring Blendor for 2 minutes in 2 ml of an ice-cold buffer containing 0.2 M sodium bicarbonate (pH 8.0), 1 percent polyvinyl pyrrolidone, and 1 mM dithiothreitol. The homogenate is filtered through cheesecloth and Miracloth and then is centrifuged at 35,000 g for 15 minutes at 4 C. The resulting supernatant is assayed for enzymatic activity.

TABLE 6. GREENING

Criteria	Critique/Comments
Test Organisms	Barley seedlings (Hordeum vulgare) are the major test organism. Pinto beans (Phaseolus vulgaris) and jack beans (Canavalia ensiformis) may also be used.
Advantages	The test is capable of identifying many different chemicals as potential toxicants. A relatively short time is required to obtain the results (chlorophyll determinations) of this assay. The test organisms are simple to grow. There is a direct relationship between the concentration of the chemicals investigated and percentage of chlorophyll inhibition. The assay can be performed by unskilled technicians.
Limitations	Specialized equipment is needed. The total time, including greening and chlorophyll determinations, is lengthly.
Response to Chemicals	Antibiotics (streptomycin, chloramphenicol) Nucleic acid analogues (5-fluorouracil, 2-thiouracil-I_{87} is 5 mM) Amino acid analogues (ethionine, p-fluorophenylalanine) Plant hormones (2,4-dichlorophenoxy acetic acid, naphthalene acetic acid, abscisic acid) Herbicides (amino triazole, paraquat, atrazine) Growth retardants (coumarin, N,N-dimethylamino succinamic acid) Ureas [diphenylurea, 3-(4-chlorophenyl)-1-(1-dimethylurea)] Fungal metabolites (alternaric acid, tentoxin) Alcohols (ethanol-I_{100} is 100%) Inorganic ions (cobalt, nickel, lead, and aluminum ions) Sugars (sucrose-I_{90} is 10 mM, fructose, glucose) Gases (carbon dioxide, carbon monoxide, methane, ethylene)
Assay Time,* hours	29, 150, 9, 8
Special Equipment	Spectrophotometer or colorimeter growth chamber
Cost*	$560
References	Arnon, 1949 Margulies, 1962 Anderson and Boardman, 1969 Keller and Huffaker, 1967 Kratky and Warren, 1971 Rubinstein et al, 1975 Borque et al, 1976 Wolf, 1977

*See time and cost explanation, pp. 9-11 in text.

RuDPCase activity is measured by incorporation of radioactive sodium bicarbonate ($NaH^{14}CO_3$) into acid-stable products in the presence of ribulose 1,5-diphosphate (RuDP). A 25-µl aliquot of enzyme is mixed on the surface of a planchet with 100 µl of reaction mixture containing the following (all are final concentrations): 100 mM Tris-HCl (pH 8.0); 50 mM $NaH^{14}CO^3$ (sp. act. 0.20 µCi/µmole); 0.3 mM RuDP; 10 mM magnesium chloride; 6 mM reduced gluthathione; 0.1 mM ethylenediaminetetraacetic acid. After 10 minutes at room temperature, the reaction is stopped by addition of 6 N acetic acid. The planchets are dried and counted in a gas-flow counter. The reaction is linear with enzyme concentration until 30 to 50 percent of the RuDP is consumed. Incorporation in the absence of RuDP is less than 2 to 3 percent of that when RuDP is added.

Details on this assay are summarized in Table 7.

Photosynthetic Oxygen Evolution

In the presence of sunlight, algae and terrestrial green plants photosynthesize and thus convert carbon dioxide and water into carbohydrates and oxygen (O_2). Even though O_2 evolution is used as a measure of photosynthesis, the O_2 evolved from a plant cell is equal to the O_2 released by photosynthesis minus the O_2 consumed by respiration. The assay described here is based on comparing the rates of O_2 evolution from algal cells pre-incubated with a test chemical to the rate of O_2 evolution from algal cells not treated with the test chemical.

The green, unicellular alga Scenedesmus is used in this assay. Scenedesmus obliquus, strain D_3, is grown in a glucose-yeast extract medium until a packed cell volume of about 10 µl/ml is obtained. About 40 ml of cells are collected and washed in 0.05 M potassium phosphate buffer (pH 6.5). The washed cells are incubated in a buffer containing concentrations of the test chemical. Following exposure to the test chemical, cells are washed free of the chemical by suspension and centrifugation. Then, 2 ml of the algal suspension is added to each of two Warburg flasks and 0.5 ml of p-benzoquinone is added to each side arm to inhibit respiration. Also, diuron is added to the side arm of the first flask (control) to inhibit photosynthesis, and water

TABLE 7. RUDP CARBOXYLASE ACTIVITY

Criteria	Critique/Comments
Test Organisms	Spinach (Spinacia oleracea), Euglena, Chlamydomonas
Advantages	The test organisms are easy to grow or obtain. A short time is required to obtain the results of this assay. There is usually a direct relationship between the concentration of the chemicals investigated and percent of enzyme inhibition.
Limitations	The enzymatic activity varies among preparations and declines with storage. The growth conditions of the plants dramatically affect enzymatic activity. Some specialized equipment is needed. The assay is performed by highly skilled technicians.
Response to Chemicals	Antibiotics (cycloheximide-I_{100} is 0.01 mg/ml; puromycin, streptomycin) Herbicides (paraquat) Growth regulators (N-(dimethylamino) succinamic acid) Arsenicals (cacodylic acid)
Assay time,* hours	2, 54, 12, 8
Special Equipment	Gas flow counter or scintillation counter
Cost*	$750
References	Keller and Huffaker, 1967 Goldthwaite and Bogorad, 1971 Rubinstein et al, 1975

*See time and cost explanation, pp. 9-11 in text.

is added to the side arm of the second flask. The flasks are equilibrated in the water bath of a Warburg apparatus at 25 C for 20 minutes. The contents of the side arms of the two flasks are then tipped into the bottom of the flasks, and immediately the measurement of O_2 evolution with high light intensity is started.

In the Warburg apparatus, volume changes are measured in an enclosed atmosphere in direct contact with the liquid under conditions in which oxygen is the only substance undergoing a net transfer between the liquid and gas phases.

Results from this manometric measurement are expressed as the percent of inhibition of O_2 evolution as a function of test chemical concentration. An oxygen electrode can be used instead of the Warburg apparatus.

Details on this assay are summarized in Table 8.

RESPIRATION

Respiration in HeLa Cells

Mitochondria are present in virtually all living cells. Both the Krebs (tricarboxylic acid) cycle and electron transport systems, the final two stages of cellular respiration, occur in the mitochondria. In these final stages, oxygen is consumed, and carbon dioxide and water are evolved. The net equation for cellular respiration is:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + \text{energy}.$$

To measure cellular respiration, it is possible to monitor either consumption of oxygen or evolution of carbon dioxide. In this assay, HeLa (human) cells are grown in Eagle's medium to a density of 4×10^5 cells/ml. Other mammalian cell types can be used, but it is not feasible to use algal cells which both photosynthesize and respire. The cells and a potential toxicant are placed in the test chamber of a precalibrated oxygen electrode, which can be purchased from a commercial source or made as described in Bruening et al (1970). The dissolved oxygen is monitored at 10 minute intervals for 1 hour. A graph of dissolved oxygen versus time is plotted, and the slope of the plot represents the rate of oxygen consumption.

TABLE 8. PHOTOSYNTHETIC OXYGEN EVOLUTION

Criteria	Critique/Comments
Test Organism	Scenedesmus obliquus, strain D[3]
Advantages	The test organism is simple to culture. Oxygen electrode can be used in place of the Warburg apparatus.
Limitations	Specialized equipment is needed. A great deal of time is required to equilibrate flasks and accurately determine gas exchange rates. This assay must be performed by skilled technicians. Variation in respiratory and photosynthetic O_2 evolution make interpretation of results difficult.
Response to Chemicals	Herbicides (1,1-dimethyl-3-phenylurea, 3-(p-chlorophenyl)-1,1-dimethylurea)
Assay Time,* hours	50, 100, 84, 11
Special Equipment	Warburg manometric apparatus
Cost*	$2330
References	Pratt and Bishop, 1968 Rubinstein et al, 1975

*See time and cost explanation, pp. 9-11 in text.

To note the effect of a potential toxicant, a different concentration of the test chemical is added to a chamber of fresh HeLa cells, and again dissolved oxygen is monitored at specific time intervals. This is carried out at several different chemical concentrations, and a graph is plotted for each concentration. Inhibition or stimulation of respiration is determined by comparing the rates of oxygen consumption for the test chemicals with those of the standard.

Toxic chemicals that inhibit respiration would most certainly influence the metabolism and viability of the organism since respiration is the process by which aerobic cells obtain energy from the oxidation of fuel molecules by molecular oxygen.

Details on this assay are summarized in Table 9.

HIGH-ENERGY PHOSPHATE PRODUCTION

Adenylate Energy Charge

Even though the production of adenosine-5' triphosphate (ATP) is a common goal of both anaerobic and aerobic metabolic activities, the measurement of ATP alone may not be an accurate index of both biomass and metabolic activity. The total energy level of the cell is dependent upon the balance between the adenosine phosphates. ATP contains two high-energy anhydride bonds, ADP contains one, and AMP none. Atkinson (1969) and Atkinson and Walton (1967) proposed an adenylate energy charge (AEC) as a fundamental metabolic control parameter:

$$AEC = \frac{ATP + 1/2\ ADP}{AMP + ADP + ATP}$$

The expression is a measure of the anhydride-bound phosphate groups per adenine moiety and is written so that the parameter will range in value from 0 to 1. In general, when the AEC > 0.5, ATP-utilizing systems increase their activities; AEC $\leq$ 0.5, ATP-regenerating sequence dominates (Atkinson, 1969; Ching et al, 1974). In this assay, cells are incubated with various concentrations of a toxicant, and the adenosine phosphates are then isolated from

TABLE 9. RESPIRATION IN HELA CELLS

Criteria	Critique/Comments
Test Organisms	HeLa (human) cells
Advantages	The test results are straightforward and easily obtainable. The test organism is easy to grow. The assay shows the effect of chemicals on a major metabolic pathway, respiration.
Limitations	This assay must be performed by skilled technicians. Only one chemical at one concentration can be tested at a time. Some special equipment is required. Reducing or oxidizing agents may interfere with and produce variation in this assay.
Response to Chemicals	Metabolic inhibitors (malonate) Detergents (Triton X-100, sodium deoxycholate)
Assay Time*, hours	30, 80, 51, 13
Special Equipment	Oxygen electrode, mammalian cell culture facilities
Cost*	$2350
References	Bruening et al, 1970

*See time and cost explanation, pp. 9-11 in text.

the cells and quantitated. This assay can be applied to bacterial, fungal, algal, mammalian, and plant cells or to mixed cultures and microbial assemblages. However, for development as an in vitro toxicity assay, the rapidly growing bacterium Escherichia coli, the green alga Euglena, or the human cell line HeLa are excellent cellular candidates.

In this assay, a known number of cells are incubated at 37 C for 1 to 2 hours with a toxicant. Adenosine phosphates are then extracted from cells with chloroform (Bostick and Ausmus, 1978; Nannipieri et al, 1978). Quantitation of ATP is based on reactions with hexokinase and glucose-6-phosphate dehydrogenase. In these enzymatic reactions, ATP causes the production of reduced nicotinamide adenine dinucleotide (NADH). NADH can then be quantitated fluorometrically down to 10^{-12} M. For determining AMP and ADP, adenylate kinase and pyruvate kinase are added to the mixture to convert these two adenosine phosphates to ATP. This ATP is then measured using the hexokinase method described above (Bostick and Ausmus, 1978). A ready-made ATP determination kit is commercially available from Calbiochem. Comparison of calculated AEC per cell values allows conclusions to be drawn on the physiological status of cell populations exposed to toxicant.

Details on this assay are summarized in Table 10.

TABLE 10. ADENYLATE ENERGY CHARGE

Criteria	Critique/Comments
Test Organisms	*Escherichia coli* is the major test organism. *Euglena* or the human cell line HeLa may also be used.
Advantages	This is a rapid indication of metabolic state. Test organism *E. coli* is commercially available and relatively inexpensive. This assay is applicable to a wide range of organisms and environmental and chemical conditions. The assay can be converted to microbially immobilized macronutrients in the microbial energy charge assay.
Limitations	Three parameters, AMP, ADP, and ATP, must be measured. The test requires a skilled technician. Analysis must be promptly performed.
Response to Chemicals	Unknown
Assay Time*, hours	8, 62, 23, 9
Special Equipment	Spectrophotometer
Cost	$920
References	Atkinson and Walton, 1967 Atkinson, 1969 Ching and Ching, 1972 Bostick and Ausmus, 1978

*See time and cost explanation, pp. 9-11 in text.

GROWTH AND CELL DIVISION

Cloning L929 Mouse Cells

The L929 cloning assay is an in vitro bioassay to examine the cytotoxic effect of a variety of toxicants. The toxicants may include particulate or soluble (aqueous or limited organic) toxicants.

The L929 cell line is carried in Eagle's Minimal Essential Medium containing 10 percent fetal calf serum, 10,000 units of penicillin per 100 ml medium, 10,000 µg of streptomycin per 100 ml medium, and 10,000 µg of mycostatin per 500 ml medium. Cells are cultured in 75 cm^2 tissue culture flasks. When cells are 75 to 90 percent confluent, 0.25 percent trypsin is used to remove cells from the flask. A 1:10 dilution of cells is made using complete media, and cells are seeded into new flasks. Cells should be split every 3 to 4 days.

A flask of L929 cells is trypsinized, and the cells are counted and diluted to 1 x 10^3 cells/ml, 8 x 10^2 cells/ml, 6 x 10^2 cells/ml, 4 x 10^2 cells/ml, and 2 x 10^2 cells/ml. The dilutions of the cells are plated onto to 60-mm dish containing 4 ml of complete medium. Twenty plates are needed at each cell concentration for one complete test. The cells are permitted to attach to the tissue culture dishes for 24 hours. The plates are then treated with various concentrations of the test chemical. Five concentrations of test chemical should be assayed in each cell dilution. Therefore, six sets of five plates should be made: one for each of the five concentrations of the test chemical plus one set to be used as an untreated control. The cells are exposed to the test chemical for 24 hours. Following the treatment period, the cells are washed twice with phosphate-buffered saline and fed normal growth medium. Microscopic examination of the plates should discern discrete colonies in approximately 10 to 12 days. At this time the plates are washed with phosphate-buffered saline, fixed with methanol, and stained by Giemsa. The colonies on the plates are counted, and a plating efficiency is determined. The plating efficiency is calculated as the number of surviving cells expressed as a percentage of the cells planted:

$$\frac{\text{\# of colonies per plate}}{\text{\# of cells seeded}} \times 100$$

An evaluation of the cytotoxic effect of the test chemical may be made by comparing the plating efficiency of the test plates with that of control plates.

Details are summarized in Table 11.

Protozoan Clonal Viability

This assay is based on the observation that when cells are subjected to toxicants or stresses, only a fraction of the population survives and reproduces. Heaf and Lee (1971) first developed this method to measure the viability of Tetrahymena after exposure to low temperatures. This viability assay is currently being adapted for toxicity testing (Persoone and Dive, 1978).

In the assay T. pyriformis is grown axenically in the dark. The cultures are then diluted to about 5 cells/ml. One ml of the dilution is placed in each well (cup) of a 100-hole, plastic, hemagglutination tray. Also, a toxicant is added in increasing concentrations to the wells containing Tetrahymena. After 6 days at 28 C, cells surviving certain toxicant concentrations will proliferate, while those affected by other toxicant concentrations will not divide. The number of wells containing growing populations, as well as the number of organisms in each well, can be counted with the naked eye and recorded. A schematic of the clonal viability test method is shown in Figure 1. The details on this assay are summarized in Table 12.

Human KB Cell Growth Rate

Nephelometric measurements, such as changes in the optical density or macromolecular complement of cell cultures, provide a basis for monitoring the growth of cell populations. In this assay, the inhibition (or possible stimulation) of mammalian cell growth is determined by measuring colorimetrically the total protein present in dividing cells both before and after incubation with a test chemical (Oyama and Eagle, 1956). Even though any of several cell lines could be employed, the rapidly growing human tumor line KB or the mouse tumor lines P388 or L1210 are excellent candidates for this assay.

TABLE 11. CLONING L929 MOUSE CELLS

Criteria	Critique/Comments
Test Organisms	L929 mouse cell line.
Advantages	The test organism is simple to culture. Several concentrations of potential toxicants may be assayed simultaneously.
Limitations	This assay must be performed by skilled technicians. Specialized equipment is needed. A very long time is required to obtain results of this assay.
Response to Chemicals	Aromatic hydrocarbons (benzene, toluene-MEC is 5 to 50 ppm) Detergents (sodium dodecyl sulfate-MEC is .005% w/v) Inorganic ions-heavy metals (cobalt, nickel-MEC is 0.11 to 1 μg/ml)
Assay Time*, hours	244, 326, 15, 9
Special Equipment	Mammalian cell culture facilities
Cost*	$750
References	Duke et al, 1977 Richardson, et al, 1977

*See time and cost explanation, pp. 9-11 in text.

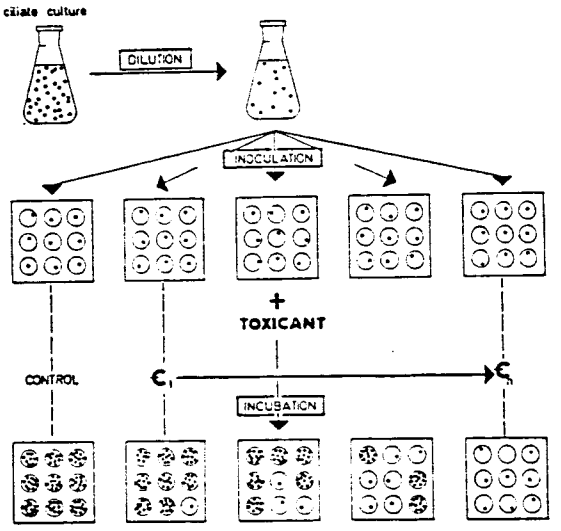

Figure 1. Schematic Representation of Viability Test Adapted to Toxicity Testing. From Persoone and Dive (1978).

TABLE 12. PROTOZOAN CLONAL VIABILITY

Criteria	Critique/Comments
Test Organism	*Tetrahymena pyriformis*
Advantages	Rapidly dividing cultures of test organisms are easily grown. Many replicates and chemical concentrations can be done simultaneously. The assay is not disrupted by particulate matter or color of the potential toxicant. The TL_{50} can be easily determined. The assay can be performed by unskilled technicians.
Limitations	A long time is required to obtain the results of this assay.
Response to Chemicals	Gases (ethylene oxide)
Assay Time*, hours	148, 210, 14, 9
Special Equipment	None
Cost*	$660
References	West et al, 1962 Heaf and Lee, 1971 Gardinono et al, 1973 Mouton and Hendrickx, 1974

*See time and cost explanation, pp. 9-11 in text.

The test is conducted as follows: KB cells, seeded at 1020 g/ml protein (2 to 3 x 10^4 cells/ml), and the appropriate concentrations of test chemical are mixed and incubated for 72 hours at 37 C. After incubation, total protein is determined in the test and untreated control cultures as described by Lowry et al (1951). For significance, untreated control cultures must go through at least six cell divisions. The number of cultures in the control group varies according to the formula $2\sqrt{n}$, where n is the test cultures or number of chemicals being tested. A positive control, cells treated with 6-mercaptopurine, exhibits an ED_{50} between .05 and 0.5 µg/ml.

Criteria for cytotoxicity of test chemicals would be any inhibition of growth caused by the test chemical. The influence of a toxicant on cell growth rate could be possibly extrapolated to the development and proliferation of tissues and organs (e.g., KB cell growth rate to nasopharynx lining proliferation).

Details of this assay are summarized in Table 13.

Human Embryonic Lung Fibroblast (WI-38) Cytotoxicity

This assay is used to measure growth-inhibition effects of various toxicants on mammalian cells.

Human embryonic lung fribroblasts (WI-38) are cultured in 75-cm^2 Falcon flasks with Eagle's Minimum Essential Medium plus mycostatin (10,000 units/500 ml medium), penicillin (10,000 units/500 ml medium), streptomycin (10,000 µg/100 ml medium), and heat-inactivated fetal calf serum (10 percent). Cells are incubated in 10 percent carbon dioxide-humidified atmosphere at 37 C. Only cells between the 15 and 35 subculture should be used.

After the cells reach about 90 percent confluence, one flask of cells is used for conducting this assay. There are eighteen 60-mm plates per assay. After assembling medium, cells, and plates, 4 ml of medium is pipetted into each plate. The stock cells in a 75-cm^2 Falcon flask are then trypsinized. After cell counts and dilutions are made, a 2 x 10^5 cells/ml suspension is seeded into each 60-mm plate.

After cells have grown to 100 percent confluence (4 to 5 days), test toxicant is added. Cells and test chemicals are then incubated for 20 hours.

TABLE 13. HUMAN (KB) CELL GROWTH RATE

Criteria	Critique/Comments
Test Organisms	Human KB cells
Advantages	The test organism is easy to culture. Many potential toxicants at several concentrations may be tested simultaneously. Inhibition of cell growth is usually an accepted standard of toxicity. National Cancer Institute routinely uses this assay for cytotoxicity screening.
Limitations	Specialized equipment is needed. A skilled technician is required to perform this assay.
Response to Chemicals	Inorganic ions-heavy metals (cadmium, nickel-MEC is 0.1 to 10 µg/ml) Nucleotide analogues (deoxy-adenosine 5'-triphosphate-MEC is $> 5 \times 10^{-5}$ M) Aromatic hydrocarbons (toluene, benzene-MEC is 1 to 5 ppm) Detergents (sodium dodecyl sulfate, Triton X-100-MEC is .001 to .005%) Carcinogenic nitrosamines (dimethyl-nitrosamine-MEC is 5 to 20 µg/ml)
Assay Time*, hours	75, 125, 12, 8
Special Equipment	Mammalian cell culture facilities, spectrophotometer
Cost*	$620
References	Oyama and Eagle, 1956

*See time and cost explanation, pp. 9-11 in text.

Following incubation, the three plates of cells per test chemical concentration (five different concentrations) are washed in phosphate-buffered saline twice and then trypsinized. The cells are collected by centrifugation at 500 g for 5 minutes. The supernatant is poured off, and 1 ml of medium is added to the centrifuge tube. The cells are suspended and counted with a hemocytometer. The average number of cells recovered from each test chemical concentration and from control plates is determined. Cell counts from the plates are averaged for all the concentrations of the test chemicals and the controls. The average counts from the test chemical plates are then expressed as a function of the number of cells obtained from the control plates. A cytotoxicity curve for the test chemical is constructed. The curve is an expression of the cellular survival as a function of concentration of the toxicant.

Effects of toxic chemicals on fibroblasts could be extrapolated to effects on human connective or pulmonary tissue.

Details on this assay are summarized in Table 14.

Mitogen Stimulation of Lymphocytes

Blastogenic transformation of lymphocytes is considered to be a manifestation of lymphocytes in cellular immunity. Measurement of the effect of test chemicals on this mitogen-induced blast transformation is a measurement of the effects on immune function. Thymus, spleen, or lymph-note cell suspensions are cultured in the presence of mitogens such as Concanavalin A (Con A), phytohemagglutinin-P (PHA), and Pokewood mitogen (PWM). Certain cells within these populations respond to the presence of mitogens by undergoing blastogenesis. The response is quantitated by monitoring ^{3}H-thymidine incorporation in mitogen stimulated and nonstimulated cultures.

Varying concentrations of the test substance are added to microlymphocyte cultures in Falcon microtest II multi-well plates. Each well is a microculture of 5×10^{5} lymphocytes growing in the presence or absence (control) of a mitogen (PHA, 50 µg/ml final; Con A, 100 µg/ml final). Each dosage of the test chemical is tested in quadruplicate with the lymphocyte cultures. Incubation is pulsed with 1 µCi of ^{3}H-thymidine. Twenty-four hours after pulsing, the cultures are harvested on glass-fiber filters using a

TABLE 14. HUMAN EMBRYONIC LUNG FIBROBLAST (WI-38) CYTOTOXICITY

Criteria	Critique/Comments
Test Organisms	Human embryonic lung fibroblasts (WI-38)
Advantages	An automatic cell counter can be used to simplify this assay. Many replicates and chemical concentrations can be tested simultaneously. The assay results are easily obtained by direct counting.
Limitations	A skilled technician with experience in microscopy is required. Some specialized equipment is needed. The possibility of human mistakes due to fatigure and boredom exists because of the tedious nature of the data collection.
Response to Chemicals	Inorganic ions-heavy metals (nickel, cadmium-MEC is 0.1 to 1 µg/ml) Gases (carbon monoxide) Aromatic hydrocarbons (benzene, toluene, ethyl benzene-MEC is 0.5 to 50 µM)
Assay Time*, hours	27, 176, 14, 9
Special Equipment	Mammalian cell culture facilities, binocular microscope (optional), hemocytometer
Cost*	$730
References	Baiile and Hardegree, 1970

*See time and cost explanation, pp. 9-11 in text.

multisample harvesting unit. ^{3}H-thymidine incorporation is determined by counting the filters, using liquid scintillation spectrophotometry.

To analyze the data, one must (1) compute mean radioactive counts per minute (cpm) and standard error of mean for all control values, i.e., PHA, Con A, and medium; (2) compute mean cpm and standard error of mean for each quadruplicate cell control, i.e., cells and medium; (3) compute mean cpm and standard error of mean for each quadruplicate PHA- and Con A-stimulated cultures; (4) for stimulation index, divide each of the quadruplicate cpm values for PHA-stimulated cells by the mean cpm value of the same cells nonstimulated, and average the four indices determined in this manner for a mean stimulation index (repeat for Con A and PHA); and (5) compare index of test-substance treated and untreated cultures.

Impairment of lymphocyte function by toxic chemicals may foreshadows the impairment of antibody formation and immune response in mammals.

Details of this assay are summarized in Table 15.

Chick Embryo Development

The assay is an attempt to predict toxicologic or teratologic (teratogenic) effects on higher vertebrates based on responses of chick embryos to potentially harmful compounds. Fertilized white leghorn eggs are candled to locate the air cell. A hole drilled through the shell over the air cell is the site of aseptic injection of 0.1 ml of a test chemical into the yolk of the developing embryo. The hole is covered with tape and the eggs are incubated at 38 C and periodically candled. Dead embryos are pathologically examined and surviving chicks are examined over a 2 to 6-week period for weight change, gross abnormalities, and mortality. At least 20 eggs are used for each chemical concentration tested to add statistical significance to the results. Eggs hatch after 21 days so the entire procedure may be performed in approximately 1 month.

Details are summarized in Table 16.

TABLE 15. MITOGEN STIMULATION OF LYMPHOCYTES

Criteria	Critique/Comments
Test Organisms	Mouse lymphocytes
Advantages	The effect of a potential toxicant is defined for a developmental parameter as well as a growth parameter. It is possible to determine a developmental change before a loss in viability or growth potential of test cells.
Limitations	Some variation of responsivenss of lymphocyte preparations may interfere with this assay. Great expense is incurred maintaining a mouse colony as a source of lymphocytes. Specialized equipment is needed. The assay must be performed by highly skilled technicians.
Response to Chemicals	Fungal toxins (aflatoxins-MEC is 5 to 20 µg/ml) Inorganic ions-heavy metals (nickel, cadmium-MEC is 0.1 to 1 µg/ml)
Assay Time*, hours	76, 76, 11, 8
Special Equipment	Scintillation counter, spectrophotometer, animal rearing facilities, mammalian cell culture facilities.
Cost*	$820
References	Savel et al, 1970

*See time and cost explanation, pp. 9-11 in text.

TABLE 16. CHICK EMBRYO DEVELOPMENT

Criteria	Critique/Comments
Test Organisms	White Leghorn chick embryos
Advantages	Large numbers involved make the results of this assay statistically meaningful. Responses of test organism correlate well with other animal responses to traditionally toxic chemicals (lead acetate, mercury II chloride). Since eggs are incubated under controlled conditions, maternal influence is not a variable, as it is in placental animals. The rapidly dividing cells may reduce the time necessary to elicit a response to possible toxicants. This assay can be performed by unskilled technicians.
Limitations	There is a lack of standardized methods in this assay. An extremely long time is required to obtain the results of this assay. Since responses are dependent on critical periods of development, responses may vary with each test chemical. One species of test organism selected, White Leghorn, may be unrealistically sensitive or insensitive to some chemicals. Response depends on several different variables: specific gravity, solubility, pH, ionic concentration, and coagulating effect. Negative results may not be significant. The lack of a placental barrier gives a questionable correlation between responses of chick embryos and responses exhibited by mammals. Specialized equipment is needed.

TABLE 16. (Continued)

Criteria	Critique/Comments
Response to Chemicals	Inorganic ions-heavy metals (lead, mercury, cobalt-LC_{71} is 0.1 mg) Food additives (monosodium glutamate, sodium benzoate) Nucleic acid analogues (5-fluorouracil) Antibiotics (tetracycline, methacycline, doxycycline) Dithiocarbamates [bis(dimethyl thiocarbamoyl)-disulfide] Organic solvents (carbon tetrachloride, n-butanol) Hallucinogens [lysergic acid diethylamide, (LSD)] Lathyrogenic agents (B-aminopropionitrile-LC_{62} is 0.63 mg) Metabolic inhibitors (2,4-dinitrophenol)
Assay Time*, hours	720 (1 month), 720, 108, 11
Special Equipment	Incubators, hatching facilities, rearing facilities, sterile injecting facilities.
Cost*	$2510
References	Feldman et al, 1958 McLaughlin et al, 1963 Gebhardt and Van Logten, 1968 Kury and Crosby, 1968 Hall, 1972 Pagnini et al, 1972 Flick et al, 1973 Messier, 1973 Palmer et al, 1973 Hulbert and Klawitter, 1974 Hall, 1976 Swartz, 1977 Zagris, 1977 Lee, 1978 Loomis, 1978

*See time and cost explanation, pp. 9-11 in text.

Trypan Blue Dye Exclusion by Human KB Cells

The ability of cultured human cells to exclude the dye trypan blue is a measure of a functioning cell membrane.

Monolayer cultures of KB cells are incubated with a test chemical for 24 hours. Following the incubation period, the cells are removed from the plates with a rubber policeman. The cell suspension is placed in 15 ml conical centrifuge tubes and centrifuged at 600 g for 5 minutes. The supernatant is discarded, and the cells are resuspended in 2 ml of phosphate buffered saline. An 0.66-ml aliquot of the cell suspension is mixed with 0.66 ml of a 0.4 percent trypan blue solution. A cell count and a viability determination are carried out for each concentration level, using a hemocytometer or cytograf. Viable cells are those cells that do not take up the trypan blue dye. Viability is calculated by:

$$\frac{\text{No. of viable cells}}{\text{total cell \#}} \times 100 = \text{percent viability}$$

A Viability Index is also calculated as follows:

$$\text{Viability Index} = \frac{\text{mean total cell count of test}}{\text{mean total cell count of control}} \times \text{mean percent of viability of test material}$$

Information from this assay may be extrapolated to other cellular or subcellular membranes because other cells are enclosed by semipermeable membranes which are structurally and functionally similar to the lipid bilayer surrounding KB cells.

Details of this assay are summarized in Table 17.

CATALYSIS (ENZYMATIC ACTIVITIES)

RNA Polymerase Activity

RNA polymerase is a multimeric enzyme that catalyzes the synthesis of RNA chains from the nucleoside triphosphates ATP, CTP, GTP, and UTP. The synthetic reaction has an absolute requirement for a divalent metal ion and

TABLE 17. TRYPAN BLUE DYE EXCLUSION BY HUMAN KB CELLS

Criteria	Critique/Comments
Test Organisms	Human KB cells
Advantages	This assay can be partially automated by the use of a hemocytometer. The test results are straightforward and easily obtainable. A relatively short time is required to obtain the results of this assay.
Limitations	Specialized equipment is needed. A skilled technician with experience in microscopy is required.
Response to Chemicals	Detergents (Triton X-100), sodium dodecyl sulfate-MEC is .001 to .005%) Polycyclic aromatic hydrocarbons (naphthalene, anthracene-MEC is 0.5 to 75 µM) Inorganic ions-heavy metals (cadmium, lead-MEC is 0.1 to 1 µg/ml)
Assay Time*, hours	30, 77, 11, 8
Special Equipment	Binocular microscope, mammalian cell culture facilities, hemocytometer
Cost*	$600
References	Corning and Firth, 1969

*See time and cost explanation, pp. 9-11 in text.

normally requires DNA as a template. RNA polymerase recognizes and binds to certain base sequences in DNA, initiates RNA synthesis, elongates the RNA chain, and finally terminates RNA synthesis with a release of a new RNA molecule.

The standard wheat germ RNA polymerase II assay mixture, in a final volume of 0.25 ml, contains 2.5 µmol of Tris-HCl (pH 7.9); 0.25 µmol of manganese chloride; 12.5 µmol of ammonium sulfate; 100 nmol each of GTP, CTP, and ATP; 1 µCi of (5-^{3}H)UTP diluted to a specific radioactivity of 1 µCi/0.1 nmol; 50 µg of heat-denatured calf thymus DNA; and 125 µg of bovine serum albumin.

The assays mixture is incubated for 15 minutes at 25 C, and the RNA is precipitated by adding 2 ml of 5 percent (w/v) ice-cold trichloroacetic acid containing 25 mM sodium pyrophosphate. After 5 minutes at 0 C, the precipitates are collected on Whatman GF/C filters and are washed under suction with five 4-ml rinses of ice-cold 2 percent trichloroacetic acid containing 10 mM sodium pyrophosphate followed by 2 ml of 95 percent ethanol. After the filters are dried under a heat lamp, they are assayed for radioactivity by liquid scintillation counting.

By changing only the template and the radioactive nucleotide, one can assay for several other polymerase activities involved in gene replication and expression. These enzymatic activities include DNA polymerase, poly(A)-polymerase, and polynucleotide phosphorylase. In all of these polymerase assays, test chemicals can be added to the reaction mixture prior to the addition of the enzyme.

By effecting RNA polymerase activity, a toxic chemical would be modifying the mechanism by which all new cellular proteins and enzymes are produced. Since RNA polymerase is present in all living organisms, results could be extrapolated to all life forms.

Details of this assay are summarized in Table 18.

Adenyl Cyclase Activity

Adenyl cyclase is a hormonally activated surface membrane enzyme which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic

TABLE 18. RNA POLYMERASE ACTIVITY

Criteria	Critique/Comments
Test Organisms	Either RNA polymerase purified from wheat germ or Escherichia coli may be used.
Advantages	RNA polymerase is present in every living organism. All assay components as well as E. coli and wheat germ enzymes are commercially available and are relatively inexpensive. Hundreds of assays can be completed daily.. Many replicates and chemical concentrations can be done simultaneously. Noting the effects of chemicals on RNA polymerase will contribute to pinpointing molecular mechanisms of chemical action. A very short time is required to obtain the results of this assay.
Limitations	Colored chemicals and precipitates interfere with this assay. A high degree of technical skill and training is necessary to assay for RNA polymerase. Specialized equipment is needed.
Response to Chemicals	Metal carcinogens (cobalt-I_{60} is 0.5 mM, lead) Antibiotics (actinomycin D, proflavine, mithramycin, rifamycin) Carcinogenic nitrosamines (dimethyl-nitrosamine, azobenzene derivatives) Polyanions (heparin, polyethylenesulfonate) Metal mutagens (lithium-I_{60} is 0.2 M) Fungal toxins (alpha-amanitin, aflatoxin) Nucleotide analogues (2'-O-methyl-adenosine 5'-triphosphate)
Assay Time*, hours	1.5, 5, 5, 8
Special Equipment	Millipore filtration manifold, liquid scintillation counter
Cost*	$530
Reference	Polya, 1973 Jendrisak and Burgess, 1975 Hoffman and Niyogi, 1977 Glazer, 1978

*See time and cost explanation, pp. 9-11 in text.

adenosine monophosphate (cAMP). The latter (cAMP) is an important regulatory molecule in both prokaryotic and eukaryotic cells.

WI-38 human diploid cells are seeded at 2 x 10^6 cells/60-mm dish. Various doses of the test chemical are added at the time of culture seeding. Cultures are incubated for 24 hours. Treated and control cells are then processed to determine adenyl cyclase activity. After trypsinization, cell pellets are obtained by centrifugation and washed three times in a 10 mM Tris buffer (pH 7.4) containing 0.1 mM dithiothreitol at 0 to 4 C. After the final wash, buffer is added followed by ice-cold magnesium chloride and sucrose to final concentrations of 3 mM and 250 mM, respectively. Better than 99 percent breakage of WI-38 cells is observed. Broken cells are subsequently centrifuged at 2000 g for 15 minutes, and the pellet is suspended in buffer containing 250 mM sucrose, 3 mM magnesium chloride, 0.1 mM dithiothreitol, and 10 mM Tris buffer (pH 7.4).

The broken cell pellet is used for the adenyl cyclase assay. Reaction mixtures contain 2 mM ATP, 6.6 mM magnesium chloride, 1.0 mM dithiothreitol, 40 mM Tris buffer (pH 7.4), and 0.05 M sucrose in a final volume of 0.5 ml. Reactions are initiated by adding 40 to 80 g of protein to the cell preparation. After incubation at 37 C for 15 minutes, the reaction is stopped by adding trichloroacetic acid to a final concentration of 5 percent. After centrifugation, the supernatant is treated three times with ethyl ether to remove the trichloroacetic acid. The solution is analyzed for cAMP using a radiotracer competitive protein binding method (commercially available). An ATP regenerating system composed of 10 mM creatine phosphate and five units of creatine phosphokinase/0.5 ml of incubation mixture is used in all experiments. Enzymatic activity is expressed as the amount of cAMP produced.

Despite the fact that cAMP plays a key role in controlling biological processes, the correlation between a chemical effect on adenyl cyclase and cellular or tissue toxicity would require extensive research and development.

Details of this assay are summarized in Table 19.

Lysosomal Enzyme Release

The destabilization of internal cellular membranes produced by toxic chemicals can be assessed by measuring the release of certain enzymes from the

TABLE 19. ADENYL CYCLASE ACTIVITY

Criteria	Critique/Comments
Test Organisms	WI-38 human diploid cells
Advantages	The assay measures a specific enzymatic activity at a surface membrane. This enzyme plays a key regulatory role in cellular metabolism. This assay assesses a specific physiological process as well as an organellar function. A relatively short time is necessary to obtain the results of this assay.
Limitations	Screening large numbers of chemicals is time consuming due to the requirement for processing cell membranes before the enzyme assay, followed by the cAMP assay. Some specialized equipment, is needed. This assay must be performed by highly skilled technicians.
Response to Chemicals	Inorganic ions-heavy metals (lead, nickel-MEC is .05-10 µg/ml) Detergents (Triton X-100-MEC is .001-.05%)
Assay Time*, hours	29, 79, 14, 9
Special Equipment	Mammalian cell culture facilities, liquid & scintillation counter
Cost*	$880
References	Klein et al, 1978

*See time and cost explanation, pp. 9-11 in text.

lysosomes into the cytoplasm. In this assay, the release of the lysosomal enzyme acid phosphatase is measured histochemically.

KB cells, seeded at 10^6 cells per 60-mm dish, are pipetted onto coverslips and then various doses of a test chemical are administered. After treatment for 24 hours, coverslips are washed in isotonic saline and then are incubated for 15 minutes in a medium of 10 mM β-glycerophosphate and 50 mM acetate buffer (pH 5.0) containing 4 mM lead nitrate and 50 mM sodium chloride. Following incubation, the coverslips are fixed for 10 seconds in one percent acetic acid, transferred to hydrogen sulfide-saturated water for 5 minutes, washed in distilled water, and mounted. Staining of acid phosphatase is indicative of damaged lysosomal membranes because intact lysosomal membranes are impermeable to the β-glycerophosphate substrate.

Other mammalian cell lines (HeLa, mouse L) can be used in this assay.

It should be noted that this assay measures the effect of a toxicant on an organellar membrane inside the cell. This assay does not measure cell lysis.

This assay measures the perturbation of an important cellular component. So, the enzymes, released from lysosomes ruptured by a toxicant, can kill cells and cause tissue necrosis. This assay can be used as quick, qualitative screen. Microspectrophotometric techniques could be used to quantitate this assay.

Details are summarized in Table 20.

Macromolecular Synthesis in KB Cells

This assay is used to assess the effects of test chemicals on the syntheses of macromolecules. The rate of incorporation of radiolabelled precursors into an acid precipitable form (macromolecular form) is used as a measure of the synthetic rate. ^{3}H-uridine incorporation is used to measure RNA synthesis, ^{3}H-thymidine incorporation to measure DNA synthesis, and ^{3}H-leucine incorporation to measure protein synthesis. The protocols for measuring any of these three parameters are the same since the radiotracer is the only variable.

TABLE 20. LYSOSOMAL ENZYME RELEASE

Criteria	Critique/Comments
Test Organisms	Human KB cells
Advantages	This assay measures a specific membrane alteration before a loss in viability occurs. The test results are straightforward and easily obtainable. A relatively short time is required to obtain the results of this assay.
Limitations	This assay requires carefully controlled conditions of incubation to prevent nonspecific lysosomal damage or staining. A skilled technician with experience in microscopy is required. Specialized equipment is needed.
Response to Chemicals	Abrasives (silica) Detergents (Triton X-100-MEC is .05-1%)
Assay Time*, hours	29, 76, 9, 8
Special Equipment	Microscope, mammalian cell culture facilities
Cost*	$560
References	Grasso et al, 1973

*See time and cost explanation, pp. 9-11 in text.

Approximately 2.0 x 10^6 KB cells are seeded into 60-mm petri dishes. Various dosages of the test chemical are added at the time of seeding of the cultures. Untreated controls are included. After 24 hours of incubation, the cultures are pulsed with 1 µCi/ml of the appropriate radiotracer (^{3}H-thymidine, ^{3}H-uridine, or ^{3}H-leucine). After a 2-hour pulse, the monolayer is rinsed with cold physiological saline, trypsinized, and resuspended in saline. Aliquots of the suspensions are taken for cell count. The remaining cells are lysed by the addition of sodium deoxycholate to a final concentration of 0.5 percent. An equal volume of cold 10 percent trichloroacetic acid is added to the suspension, and the resulting precipitate is collected on glass-fiber filters. The filters are then dried, and the radioactivity is determined by liquid scintillation spectrophotometry. The results are calculated as counts per minute of isotope incorporated per cell.

This assay could be combined and correlated with other biochemical or enzymatic assays. For example, the chemical inhibition of cellular RNA synthesis could be correlated with the effect of that chemical on RNA polymerase activity. Also, this assay protocol can be expanded to monitor other cellular syntheses by simply utilizing other labelled precursors (e.g., ^{3}H-acetate for fatty acid synthesis).

Details of this assay are summarized in Table 21.

OTHER CELLULAR PROCESSES

Cyclosis

Cyclosis or protoplasmic streaming is the regular, cyclic movement of particles within a cell. Lucas (1977) has devised an assay to measure the inhibition of cyclosis caused by various levels of ammonium sulfate. This assay could be adapted for toxicity testing.

In this procedure, internodal cells of the alga Chara are cut from an algal mat 1 day prior to the experiment. After cutting, the cells are soaked in 1.0 mM sodium bicarbonate buffer (pH 9.0) and are subjected to a regime of 13 hours of light and then 11 hours of dark. All cells are illuminated under fluorescent lights (10 W/sqm) for 2 hours before the start of an experiment.

TABLE 21. MACROMOLECULAR SYNTHESIS IN KB CELLS

Criteria	Critique/Comments
Test Organisms	KB Human cell cultures
Advantages	This assay measures specific functional properties of cells and can be indicative of functional alteration or loss before general toxic effects (such as death) are observed. A relatively short time is required to obtain the results of this assay.
Limitations	This assay must be performed by skilled technicians. Specialized equipment is needed.
Response to Chemicals	Inorganic ions-heavy metals (nickel, lead-MEC is 0.1 to 10 µg/ml) Polycyclic hydrocarbons (naphthalene-MEC is 1 to 50 µM) Detergents (hexachlorophene-MEC is 5 to 100 µg/ml)
Assay Time*, hours	34, 78, 11, 8
Special Equipment	Liquid scintillation counter, mammalian cell culture facilities
Cost*	$720
References	Carr and Ligaton, 1973

*See time and cost explanation, pp. 9-11 in text.

At the beginning of the test, cells are soaked for 1 hour in a solution of the chemical to be tested. After exposure to the chemical, cells are examined, and cyclosis is measured with a binocular microscope having an ocular micrometer. The time required for a standard-size cytoplasmic particle to traverse 1000 μm is measured with a stopwatch. Rates of cyclosis are measured in 10 cells and are expressed as the mean ± standard error.

It would be difficult to correlate a specific chemical effect on algal cyclosis with a chemical effect on metabolic processes in mammalian cells and tissues.

Details on this assay are summarized in Table 22.

Hemolysis

This bioassay is capable of identifying the hemolytic effect of various potential toxicants. To measure this hemolytic effect, solutions of varying concentrations of the suspected toxicant, and a buffered saline solution containing 0.1 mM ethylenediaminetetraacetate (EDTA), are prepared and washed rat erythrocytes are added. The concentration of these cells should be 0.5 percent (volumetrically). The solutions are incubated for 1 hour and then centrifuged for 10 minutes at 1000 g to remove intact red cells. The supernatant fraction is then spun for 15 minutes at 20,000 g to remove any remaining particulate matter. The optical densities of the final supernatant fractions are measured at 542 nm to estimate hemoglobin. Addition of a like amount (0.5 percent) of red blood cells to water gives the value for 100 percent hemolysis.

Information from this assay may be extrapolated to other cellular and subcellular membranes because other cells are enclosed by semipermeable membranes which are structurally and functionally similar to the lipid bilayer surrounding erythrocytes. Also, since erythrocytes contain hemoglobin which transports oxygen to tissues, any erythrocyte aberation would cause detrimental effects elsewhere in the body.

Details on this assay are summarized in Table 23.

TABLE 22. CYCLOSIS

Criteria	Critique/Comments
Test Organisms	Chara corallina is the major organism. Nitella translucens and Elodea may also be used.
Advantages	None
Limitations	Inhibition of cyclosis goes from 0 to 100 percent with a small change in the concentration of certain interfering chemicals. The test organism is very difficult to culture. A skilled technician experienced in microscopy is necessary. A relatively long time is required to obtain the results of this assay. Some chemicals may cause increased, instead of decreased streaming. Only one assay at one test chemical concentration can be performed in one hour. There is a variable rate of streaming which is dependent on cell volume. Specialized equipment is needed.
Response to Chemicals	Metabolic inhibitors (2,4-dinitrophenol). Chlorinated aliphatics (chloroform). Inorganic salts (ammonium sulfate- I_{25} is 0.5 mM). Gases (oxygen) Sugars (mannitol, sucrose)
Assay Time*, hours	30, 141, 58, 11
Special Equipment	Binocular microscope with ocular micrometer
Cost*	$1810
References	Pfeffer, 1938 Thaine, 1964 Geis and Morrison, 1971 Lucas, 1977

*See time and cost explanation, pp. 9-11 in text.

TABLE 23. HEMOLYSIS

Criteria	Critique/Comments
Test Organisms	Rat or human erythrocytes
Advantages	This assay can be performed by an unskilled technician. A very short time is required to obtain the results of this assay. The test results are straightforward and easily obtainable. Erythrocytes from several sources are commercially available. Many chemicals and varying chemical concentrations may be tested simultaneously.
Limitations	Rat and human erythrocytes vary from batch to batch because of nutritional and genetic differences in donors. With some chemicals it is difficult to establish a dose-response relationship.
Response to Chemicals	Sulfhydryl inhibitors (p-chloromercuribenzoic acid-EC_{40} is 37.4 mg) Inorganic ions-heavy metals (lead, mercury) Hormones (epinephrine, prostaglandin E_2) Peroxides (peroxidized microsomal lipids, hydrogen peroxide) Abrasives (silica) Arylhydrazines (phenyldrazine-EC_{77} is 100% solution, m-toylhydrazine) Detergents (Triton X-100) Inorganic ions-halides (iodide) Ionic surfactants (alkyltrimethylammonium halides) Chlorinated antibacterials (hexachlorophene) Industrial particulates (asbestos) Buffers (Tris-HCl-EC_{100} is 100% solution)
Assay Time*, hours	2, 4, 4, 8
Special Equipment	Spectrophotometer or colorimeter
Cost	$440

TABLE 23. (Continued)

Criteria	Critique/Comments
References	Sheets et al, 1956 Allen and Rasmussen, 1971 Lessler and Walter, 1973 Itano et al, 1974 Klebanoff and Clark, 1975 Luthra et al, 1975 Majer, 1975 Light and Wei, 1977 Summerton et al, 1977 Pesh-Iman et al, 1978 Zaslavsky et al, 1978

*See time and cost explanation, pp. 9-11 in text.

Protozoan Vacuole Contraction

This assay is based on the observations that contractile vacuoles function in regulating osmotic pressure (Rifkin, 1973) and expelling waste substances from the cell. Nilsson (1974) developed an assay to measure the effect of a foreign substance on vacuolar contraction in Tetrahymena. Under favorable conditions, the vacuole contracts at regular intervals. However, when the protozoan is stressed, the timing of the intervals may be altered.

In the assay, T. pyriformis is grown axenically in an enriched medium. Aliquots of the cells are then incubated at 28 C in the test chemical solution. Observations are made using a light microscope, a Reichert anoptral optical system or a similar viewing system, during the 1-hour period, with expulsion intervals of vacuoles from several cells being recorded. The time required to reach normal size and the expulsion intervals for control cells are also recorded. The time intervals for individual cells are recorded separately.

It would be difficult to correlate results from this assay with data obtained from mammalian cells and tissues. However, if vacuolar contraction were developed as a toxicity assay, those results could complement data obtained from the protozoan clonal viability assay and the protozoan motilty assay.

The use of protozoans as test organisms in toxicity studies would appear to bridge the gap between undifferentiated prokaryotic organisms, such as bacteria, and the more complex metazoa (Woodard, 1976).

Details on this assay are summarized in Table 24.

Protozoan Motility

Bergquist and Bovee (1974) conceived an original method for measuring the motility of ciliates by microphotography.

Tetrahymena pyriformis is centrifugally pelleted and separated from axenic growth medium, washed, and again pelleted centrifugally. Then it is introduced by pipette into a holding chamber. For the assay, greater than 98 percent of the organisms should be motile. The test chamber is covered at its

TABLE 24. PROTOZOAN VACUOLE CONTRACTION

Criteria	Critique/Comments
Test Organisms	Tetrahymena pyriformis
Advantages	The test organism is easy to obtain commercially and culture. The effects of potential toxicants can be easily observed. Vacuolar contraction can be standardized by controlling temperature, the age of cells, and the nutritional state of cells.
Limitations	Response times may vary even in a single cell. A long time is required to obtain the results of this assay. The assay must be performed by skilled technicians with experience in microscopy. Vacuolar contraction varies with vacuole size and a cell may contain more than one vacuole. Numerous observations must be made.
Response to Chemicals	Dipolar solvents (dimethyl sulfoxide)
Assay Time*, hours	17, 143, 30, 10
Special Equipment	Light microscope, temperature control unit
Cost*	$1130
References	Rifkin, 1973 Nilsson, 1974 Patterson and Sleigh, 1976

*See time and cost explanation, pp. 9-11 in text.

bottom by #5 Whatman filter paper and suspended in 200 ml of test solution. The pH is monitored and readjusted to 7.0 as needed. After 1 hour, samples are pipetted onto clean slides for microscopic examination. Multiple-exposure photographs are taken stroboscopically using a perforated aluminum disc attached to a stirring motor, which is equipped with a variable-speed reduction gear, permitting optimal image-spacing. The revolving perforated disc is interposed between a Zeiss RA microscope and the removable light, permitting full use of the microscope's optics and lighting. Negative-image films are then projected onto a frosted glass screen and maximal-speed paths and linear spacings are measured. As an end point, the distances between the ciliates in the multiexposed photomicrographs are measured very easily by projection of the negatives onto a large screen. This method permits large data samples to be obtained quickly and easily. The results are then tested statistically for comparative and descriptive purposes. This assay might be automated by employing a computer and a TV.

A chemical inhibition of protozoan flagellar or ciliary function might be similar to effects on mammalian tracheal tissues. The advantage of using protozoans in toxicity studies is mentioned in the description of the vacuole contraction assay.

Details on the assay are summarized in Table 25.

Phagocytosis by Alveolar Macrophages

This assay employs a primary cell line, alveolar macrophages, to define the acute cellular toxicity of particulates and other chemicals. Toxic effects are assessed by measuring a macrophage function, phagocytosis.

Rabbit alveolar macrophages are harvested. The cellular composition should contain a minimum of 95 percent alveolar macrophages. The cell suspension is then adjusted to a concentration of 1×10^6 cells/ml.

One-ml aliquots of the cell suspension are placed in 60-mm tissue culture plates and the macrophages allowed to adhere. After 2 hours, the medium is decanted and 5 ml of fresh medium is added to each plate.

The test chemical is diluted to the desired concentration with cell culture medium and 1-ml aliquots are added to each plate. Initially, three

TABLE 25. PROTOZOAN MOTILITY

Criteria	Critique/Comments
Test Organisms	Tetrahymena pyriformis is the major test organism. Paramecium caudatum may also be used.
Advantages	The test organisms are easy to culture. The decrease in motility is usually related to the concentration of the potential toxicant. Large data samples may be easily obtained.
Limitations	Precipitates or particulate matter may interfere with this assay. Specialized equipment is needed. There is a lengthy film development period in microphotography. This assay requires a skilled technician with experience in microscopy.
Response to Chemicals	Heavy metals (nickel, cadmium) Ionic detergents (sodium dodecylsulfate, sodium stearate)
Assay Time*, hours	23, 136, 27, 9
Special Equipment	Microphotography unit
Cost*	$1000
References	Andrivon, 1968, 1972 Dryl and Bujwid-Cwik, 1972 Berquist and Bovee, 1973, 1974 Perkins and Cieresko, 1973

*See time and cost explanation, pp. 9-11 in text.

concentrations of test chemical are used with three replicates per dose. The plates are incubated in a humidified 95 percent air, 5 percent carbon dioxide atmosphere at 37 C for 20 hours. The plates may be rotated for the first hour to ensure uniform exposure of the test material. At the end of the incubation period, the medium is decanted and fresh medium added.

Phagocytic activity is measured by addition of 1.1 μm polystyrene latex particles to alveolar macrophages cultured in Lab-Tek four-chamber microslides (approximately 25 particles/cell in 1 ml of medium). One hour after the addition of latex particles, the slides are then exposed for an additional 5 to 6 minutes with 1:1 aqueous dilution of Wright's stain. After air drying, the slides are placed in xylene for one hour to dissolve extracellular particles. Following an additional drying step, the slides are mounted with permount. Phagocytic activity is determined under oil immersion by scoring a minimum of 200 cells. Each cell that contains at least one particle is considered phagocytically active. Typically, 80 to 90 percent of the cells in control cultures ingest one or more particles.

This assay is already in limited use as a toxicity assay.

Details on this assay are summarized in Table 26.

Amino Acid Transport

A measurement of plasma membrane function is active transport and exchange of molecules into and out of the cell. This assay tests membrane function by measuring the active transport of the amino acid histidine into KB cells.

For this assay, 10^6 KB cells in 3 ml of suspension culture medium are exposed to various doses of the test chemical for 24 hours. At the end of the exposure period, the cells are washed and then suspended in incubation medium containing 131 mM sodium chloride, 5.2 mM potassium chloride, 1.3 mM magnesium sulfate, and 1.0 mM calcium chloride in 10 mM sodium phosphate buffer (pH 7.4). One μCi of ^{3}H-histidine is added, and the mixture is incubated with agitation at 37 C. Samples are taken every minute for 5 minutes for radioactivity and cell number determinations. For measurements of histidine uptake, the cells in each sample are washed in cold saline to remove

TABLE 26. PHAGOCYTOSIS BY ALVEOLAR MACROPHAGES

Criteria	Critique/Comments
Test Organisms	Rabbit alveolar macrophages
Advantages	The assay measures a functional process of macrophages, phagocytosis, and indicates specific functional alterations produced by test substances. A relatively short time is required to obtain the results of this assay. Alterations in phagocytosis occur before any general loss in cellular viability.
Limitations	A great expense is incurred maintaining a rabbit colony as a source of macrophages. The assay can be performed by skilled technicians. Other specialized equipment is needed. The assay is labor intensive in the preparation of macrophages and in monitoring results. Macrophage preparations may vary in responsiveness from day to day.
Response to Chemicals	Gases (cigarette smoke, fly ash-MEC is 10 to 100 µg/ml) Inorganic ions-heavy metals (cadmium, zinc-MEC is 25 to 100 mM)
Assay Time*, hours	27, 27, 17, 9
Special Equipment	Microscope, animal rearing facilities, mammalian cell culture facilities
Cost*	$890
References	Green and Carolin, 1967 Duke et al, 1977

*See time and cost explanation, pp. 9-11 in text.

extracellular histidine. The total radioactivity in the cells is determined by liquid scintillation counting. Rates of histidine transport are then determined and compared by plotting the concentration of intracellular, labelled histidine against the extracellular concentration by the classic method of Lineweaver and Burke. Toxicity is indicated by decreases in the rate of histidine transport as compared to untreated controls.

Information from this assay may be extrapolated to other cellular or subcellular membranes because other cells are enclosed by semipermeable membranes which are structurally and functionally similar to the lipid bilayer surrounding KB cells.

Details on this assay are summarized in Table 27.

OTHER POTENTIAL PHYSIOLOGICAL TOXICITY ASSAYS

Several other assays show promise as toxicity screens and are critiqued below. In each case, few data on the assay or on effects of known toxicants exist. For the chlorophyll fluorescence assay, much of the information is in press and will appear in the near future. However, it is impossible for us to develop a complete protocol for these assays with the information presently available.

The Microtox bacterial luminescence assay designed by Beckman Instruments, Carlsbad, California, shows promise as a potential toxicity screen.

All living organisms have certain structural and metabolic similarities. It is often possible to extrapolate studies performed on one organism to other living systems because of these similarities. Cells of luminescent bacteria are structurally and functionally similar to other living cells. The semipermeable membranes surrounding all cells are quite similar. In addition, certain metabolic processes (e.g., respiration) are common to all cells.

According to Beckman, the Microtox system is simple, rapid, inexpensive, and accurate. From the data available, this appears to be true. Preliminary test results also seem to correlate with results of the 96-hour fish acute toxicity test. Although this assay appears to be an effective toxicity screen, the methods and results still need to be validated by

TABLE 27. AMINO ACID TRANSPORT

Criteria	Critique/Comments
Test Organisms	Human KB cells
Advantages	Simple kinetics (one or two points) could allow time for several chemical concentrations to be tested simultaneously. The test organism is easy to culture. The assay can detect alternations in a specific membrane process before generalized toxicity. A relatively short time is required to obtain the results of an assay.
Limitations	If detailed kinetic measurements are made, only one concentration of one specific chemical can be tested at a time. A highly skilled technician is required to perform this assay. Many measurements are required to obtain the results of this assay. Some specialized equipment is required.
Response to Chemicals	Detergents (Triton X-100, sodium deoxychloate-MEC is .001 to .05%) Inorganic ions-heavy metals (nickel, cadmium-MEC is 10 to 50 mM) Inorganic salts (magnesium chloride, calcium carbonate)
Assay Time*, hours	29, 53, 11, 8
Special Equipment	Liquid scintillation counter, mammalian cell culture facilities
Cost*	$810
References	Matthews et al, 1970

*See time and cost explanation, pp. 9-11 in text.

independent researchers. After significant testing with known toxicants, an objective decision can be made about the utility of the Microtox system.

Another promising assay is the chlorophyll fluorescence assay developed by Arntzen, Steinback, and others at the USDA laboratory at the University of Illinois, Urbana.

Fluorescence of living leaves has been known for over 100 years, having been recognized by Muller in 1874 as a pathway of energy dissipation which competes with energy utilization in photosynthesis and with heating of the leaf. Since Muller's time, studies of in vivo chlorophyll fluorescence in algae, in leaves, and in chloroplasts of higher plants have improved our present understanding of the light reactions of photosynthesis.

Quite recently, in vivo chlorophyll fluorescence measurement has also begun to be recognized as a means of detecting damage in intact plants subjected to environmental stresses and deleterious agents. The technique is an attractive one because data collection is easy, fast, and nondestructive, and can be done in the field. The potential applications of fluorescence measurement at the whole plant level probably will not be limited to detection of stress-induced damage but may be extended to use as a versatile diagnostic tool in plant pathology and as a screening tool in plant genetics. This fluorescence assay could be used to complement results from the Hill reaction and/or greening assays and to provide a broad data base about toxic effects on plants.

McFarlane, Rogers, and Bradley at U.S. EPA, Environmental Monitoring and Support Laboratory, Las Vegas, are developing a rapid toxicity assay involving tritium oxidation by soil microorganisms. In this assay, water is added to air-dried soil in a reaction vessel, and the slurry is incubated overnight. Tritium is injected into the reaction vessel and, after a fixed time, the amount of tritium oxidized to water is determined using a liquid scintillation spectrophotometer. Toxicity is determined by adding a fixed amount of potential toxicant to the reaction vessel prior to the addition of the tritium. The oxidation rate of this test sample is compared with that of the standard (untreated) sample. Even though several chemicals (e.g., silver nitrate, monuron, cadmium chloride) have been tested using this assay, the test organisms are not standardized, and different soil samples may contain

different populations of microbes. This toxicity assay could probably be used only as a pretest to indicate the need for further testing.

From the limited available information, these assays appear to be excellent candidates for use as toxicity assays. Since we do not have sufficient information to evaluate them by the same criteria used to evaluate other assays in this report, any decision about their utility as toxicity screens would be premature. However, it appears that little laboratory development of these assays would be necessary. As more information on these assays becomes available, objective ratings will be possible.

TABULAR COMPARISON OF CRITERIA

On the following pages are five tables (Tables 28 through 32) which show the relative strengths and weaknesses of these 24 physiological assays. These tables provide, at a glance, comparative data on which specific recommendations are based (see Discussion and Recommendations). With the exception of the "data base" category (Table 32), the criteria used in these five tables are defined and discussed in the introduction. The "data base" category in Table 32 refers to the relative amount of data available about chemical effects on a physiological process. The data base for each assay was rated as good (+++), fair (++), or poor (+). This is merely a subjective rating based upon sources revealed during our literature search (see Appendix).

TABLE 28. SOURCE OF TEST ORGANISM

Assay	Culture or grow	Animal facility	Commercially prepared
Acetylene Reduction	X		
Hill Reaction	X		
Greening	X		
RuDP Carboxylase Activity	X		
Photosynthetic Oxygen Evolution	X		
Respiration in HeLa Cells	X		
Adenylate Energy Charge	X	X	X
Cloning L929 Mouse Cells	X		
Protozoan Clonal Viability	X		
Human (KB) Cell Growth Rate	X		
Human Embryonic Lung Fibroblast (WI-38) Cytotoxicity	X		
Mitogen Stimulation of Lymphocytes		X	
Chick Embryo Development		X	
Trypan Blue Dye Exclusion by Human KB Cells	X		
RNA Polymerase Activity	X		X
Adenyl Cyclase Activity	X		
Lysosomal Enzyme Release	X		
Macromolecular Synthesis in KB Cells	X		
Cyclosis	X		
Hemolysis		X	X
Protozoan Vacuole Contraction	X		
Protozoan Motility	X		
Phagocytosis by Alveolar Macrophages		X	
Amino Acid Transport	X		

TABLE 29. TEST ORGANISMS AND ORGANISMAL LEVEL OR PARAMETER EVALUATED

Assays	Test organism						Level or parameter			
	Human	Other vertebrate	Protozoan	Higher plant	Alga	Bacteria	Organismal	Cellular	Organellar	Enzymatic
Acetylene Reduction					X	X			X	
Hill Reaction				X	X				X	
Greening				X			X			
RuDP Carboxylase Activity				X	X					X
Photosynthetic Oxygen Evolution					X				X	
Respiration in HeLa Cells	X							X		
Adenylate Energy Charge	X	X		X	X	X		X		
Cloning L929 Mouse Cells		X						X		
Protozoan Clonal Viability			X					X		
Human KB Cell Growth Rate	X							X		
Human Embryonic Lung Fibroblast (WI-38) Cytotoxicity	X							X		
Mitogen Stimulation of Lymphocytes		X						X		
Chick Embryo Development		X					X			
Trypan Blue Dye Exclusion by Human KB Cells	X							X		
RNA Polymerase Activity				X		X				X
Adenyl Cyclase Activity	X									X
Lysosomal Enzyme Release	X								X	
Macromolecular Synthesis in KB Cells	X									X
Cyclosis					X			X		
Hemolysis	X	X						X		
Protozoan Vacuole Contraction			X					X		
Protozoan Motility			X					X		
Phagocytosis by Alveolar Macrophages		X						X		
Amino Acid Transport	X							X		

TABLE 30. SPECIAL EQUIPMENT

Assays	Centrifuge	Microscope	Spectrophotometer	Scintillation Counter	Mammalian cell culture facilities	Other equipment
Acetylene Reduction						Gas chromatograph
Hill Reaction	X		X			
Greening	X		X			
RuDP Carboxylase Activity	X			X		
Photosynthetic Oxygen Evolution						Warburg apparatus
Respiration in HeLa Cells					X	Oxygen electrode
Adenylate Energy Charge			X			
Cloning L929 Mouse Cells					X	
Protozoan Clonal Viability						
Human (KB) Cell Growth Rate			X		X	
Human Embryonic Lung Fibroblast (WI-38) Cytotoxicity	X	X			X	Hemocytometer
Mitogen Stimulation of Lymphocytes			x	x	X	Animal rearing facilities
Chick Embryo Development						Incubators, hatching and rearing facilities, sterile injecting facilities
Trypan Blue Dye Exclusion by Human KB Cells	X	X			X	Hemocytometer
RNA Polymerase Activity				X		*Millipore filtration* manifold
Adenyl Cyclase Activity	X			X	X	
Lysosomal Enzyme Release		X			X	
Macromolecular Synthesis in KB Cells				X	X	
Cyclosis		X				
Hemolysis	X		X			
Protozoan Vacuole Contraction		X				
Protozoan Motility	X					Microphotography unit
Phagocytosis by Alveolar Macrophages		X			X	Animal rearing facilities
Amino Acid Transport				X	X	

TABLE 37. TIMES COSTS, AND TECHNICIAN SKILL

Assay	Assay Set time*, hours	Total assay set time**, hours	Total technician time†, hours	Technician Skill‡	Other (administrative) time§, hours	Other costs and consideration	Approximate total cost#, $
Acetylene Reduction	2.5	54	12	++	8		620
Hill Reaction	3	57	18	+	11		820
Greening	29	150	9	+	8	$35 (greenhouse fee)	560
RuDP Carboxylase Activity	2	54	12	+++	8		750
Photosynthetic Oxygen Evolution	50	100	34	++	11		2330
Respiration in HeLa Cells	30	80	51	++	13		2350
Adenylate Energy Charge	8	62	23	++	9		920
Cloning L929 Mouse Cells	244	326	15	++	9		750
Protozoan Clonal Viability	148	210	14	+	9		660
Human (KB) Cell Growth Rate	75	125	12	++	8		620
Human Embryonic Lung Fibroblast (WI-38) Cytotoxicity	27	176	14	++	9		730
Mitogen Stimulation of Lymphocytes	76	76	11	+++	8	$100 (animal facility fee and rearing	820
Chick Embryo Development	720 (1 month)	720	108	+	11	$150 (animal facility fee and rearing)	2510
Trypan Blue Dye Exclusion by Human (KB) Cells	30	77	11	++	8		600
RNA Polymerase Activity	1.5	5	5	+++	8		530
Adenyl Cyclase Activity	29	79	14	+++	9		880
Lysosomal Enzyme Release	29	76	9	++	8		560
Macromolecular Synthesis in KB Cells	34	78	11	++	8		720
Cyclosis	30	141	58	++	11	$30 (special algal culture facilities)	1810
Hemolysis	2	4	4	+	8		440
Protozoan Vacuole Contraction	17	143	30	++	10		1130
Protozoan Motility	23	136	27	++	9		1000
Phagocytosis by Alveolar Macrophages	27	27	17	++	9	$100 (animal facility fee and rearing)	890
Amino Acid Transport	29	53	11	+++	8		810

*Three replicates of five chemical concentrations.

**Including cell growth, solution preparation, organelle or enzyme preparation, and data recording.

†Including GLP, running assay, and solution preparation.

‡Rated from + (unskilled) to +++ (highly skilled).

§Including Ph.D. supervision, managerial time, data analysis, and reporting.

#Costs are estimated for the purpose of comparison only. Actual costs may vary to 20 to 25 percent from these figures at different laboratories.

TABLE 32. DATA BASE AND COMMENTS

Assay	Data base*	Comments
Acetylene Reduction	++	Acetylene gas is very explosive.
Hill Reaction	+++	Chloroplasts and activities vary
Greening	+++	This assay has already been used to test many chemicals.
RuDP Carboxylase Activity	+	This assay is rapid and relatively inexpensive, but activity varies.
Photosynthetic Oxygen Evolution	+	Respiratory and photosynthetic rates may vary.
Respiration in HeLa Cells	+	An extensive time and great cost are required to complete an assay set.
Adenylate Energy Charge	++	No chemical effects on this assay are known, but it measures a universal metabolic process.
Cloning L929 Mouse Cells	++	A long time is required to complete an assay set.
Protozoan Clonal Viability	++	A long time is required to complete an assay set, but many assays can be performed simultaneously.
Human (KB) Cell Growth Rate	++	This assay has already had limited use as a toxicity screen.
Human Embryonic Lung Fibroblast (WI-38) Cytotoxicity	+++	Toxic chemical effects could be extrapolated to human pulmonary tissues.
Mitogen Stimulation of Lymphocytes	+	A great expense is incurred in maintaining a mouse colony.
Chick Embryo Development	+++	Extensive time and great cost are required to complete an assay set. There is no standardized endpoint.
Trypan Blue Dye Exclusion by Human KB Cells	+	Results from this assay can be extrapolated to all membranes.
RNA Polymerase Activity	++	Hundreds of assays can be completed daily and correlated to all life forms.
Adenyl Cyclase Activity	+	It is difficult to correlate assay results with cellular or tissue toxicity.

TABLE 32. (Continued)

Assay	Data base*	Comments
Lysosomal Enzyme Release	+	Results from this assay can be extrapolated to cellular and tissue levels.
Macromolecular Synthesis in KB Cells	++	This assay could be combined with RNA polymerase activity to detect chemical inhibition of RNA synthesis.
Cyclosis	+	This assay has little relevance to mammalian cell and tissues.
Hemolysis	+++	The assay is rapid, inexpensive, and results can be extrapolated to many systems.
Protozoan Vacuole Contraction	+	Results may be correlated to other assays involving protozoans.
Protozoan Motility	++	A long time and great expense is required to complete an assay set.
Phagocytosis by Alveolar Macrophages	+++	This assay is already in limited use as a toxicity screen.
Amino Acid Transport	+	The results of this assay can be extrapolated to other membranes.

*Rated +++ (good), ++ (fair), and + (poor).

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APPENDIX

LITERATURE SEARCH METHODS

APPENDIX

LITERATURE SEARCH METHODS

LITERATURE SEARCH

To obtain information on physiological assay methods and on the effects of potential toxicants on these assays, manual literature searches of published articles, reviews, abstracts, and bibliographies as well as computerized searches of selected data bases were conducted. This literature search was conducted over a period of 6 weeks from May 7, 1979, to June 15, 1979, by the methods outlined in Van Voris, et al (1979).

Hand Searches

In the hand literature searches, 50 of the most widely used and subscribed journals in biochemistry, molecular biology, enzymology, cell biology, pharmacology, botany, zoology, toxicology, ecology, and microbiology were scanned over the past year (May, 1978-April, 1979) for references pertaining to rapid physiological toxicity assays (Table A-1). Additional references were located by reviewing the abstract and bibliographic publications Bibliography of Agriculture, Environmental Abstracts, Biological Abstracts, and Chemical Abstracts. These bibliographic publications were searched over a 5-year period (usually 1974-1979).

The applicability of the indexed articles was based upon the titles only, or the abstracts when available. In many instances it was necessary to make subjective decisions as to whether an article pertained to in vitro physiological assays, particularly when abstracts were not available. Additional references were reviewed on the basis of bibliographic citations in individual papers. Several articles familiar to the authors of this report were also reviewed.

Computer Searches

Computer searches were conducted on data bases simultaneously with the hand searches. The rationale of the data bases selected was to give coverage to all types of literature reporting ecological effects of toxic chemicals. The bases searched were:

- Bioscience Information System (BIOSIS) which focuses on life sciences worldwide since 1964. The data base is journal publications containing the entire life sciences and including microbiology, plant and animal sciences experimental medicine, agriculture, pharmacology, ecology, bioengineering, biochemistry, and biophysics. The producer is BioSciences Information Service of Biological Abstracts.
- Toxicology Information On-line (TOXLINE) which gives worldwide coverage to toxicology studies on animals and humans since 1971. The data base includes toxicology studies on environmental pollutants and chemicals, adverse drug reactions, and other toxic materials. It is produced by the National Library of Medicine.

BIOSIS, searched from 1969 to the present, printed only titles. We found this data base to be particularly useful because it has controlled vocabularies with articles referenced by both key words and topics (e.g., Hill reaction and photosynthesis) so that all references pertaining to a desired subject under a topic are printed. TOXLINE was the more comprehensive data base (searched since 1971), overlapping somewhat with BIOSIS.

The information retrieved from computer searches is dependent on words entered into the computer by the user as well as the key word descriptors used by authors or reviewers of articles used in the different data bases. In many cases, the key words used by a reviewer omit important facets of the article or refer to lightly covered topics because of misleading titles. The key words to be entered in the computer by the searchers were determined by assay title, test organism, and cellular processes involved. Key words (assays, organism, and processes) were taken from Table 2 of this report. For example, for protozoan vacuole contraction, the key words protozoan and contractile vacuole were among those entered in the computer. Articles that appeared relevant were then reviewed and specific information was extracted from them.

TABLE A-1. JOURNALS SCANNED IN LITERATURE SEARCH

Agricultural and Biological Chemistry	FEBS Journal
Analytical Biochemistry	Food and Cosmetics Toxicology
Annual Review of Microbiology	Histochemistry and Cytochemistry
Applied and Environmental Microbiology	Journal of Bacteriology
Biochemical and Biophysical Research Communications	Journal of Biological Chemistry
The Biochemical Journal	Journal of Cell Biology
Biochemistry	Journal of Cell Science
Biochimica et Biophysica Acta (Nucleic Acids, Enzymology, Reviews on Cancer, Lipids, Bioenergetics)	Journal of Cellular Physiology
Botanical Gazette	Journal of Experimental Botany
Botanical Review	Journal of General Microbiology
Canadian Journal of Biochemistry	Journal of Molecular Biology
Carbohydrate Research	Journal of Protozoology
Cell	Methods in Enzymology
Developmental Biology	Molecular and General Genetics
Ecology	Molecular Pharmacology
Ecotoxicology and Environmental Safety	Nature
Environmental Science and Technology	Nucleic Acids Research
European Journal of Biochemistry	Parasitology
Experimental Cell Biology	Pesticide Biochemistry and Physiology
Experimental Cell Science	
Experientia	

TABLE A-1. (Continued)

Physiologia Plantarum
Plant Physiology
Plant Science Letters
Planta
Proceedings of the National Academy of Science
Proceedings of the Society for Experimental Biology and Medicine
Sabouraudia
Science
Toxicology and Applied Pharmacology
Virology
Weeds

REFERENCE

Van Voris, P., S. Pomeroy, H. Grotta, and A. Rudolph. March, 1979. Literature Evaluation of Field-Observed Effects of Toxic Chemicals. OPTS/EPA Contract No. 68-01-5043. 27 pp.

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15. SUPPLEMENTARY NOTES

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16. ABSTRACT

Battelle's Columbus Laboratories has contracted with the Office of Pesticides and Toxic Substances, U.S. Environmental Protection Agency, to develop a list of physiological assays as potential toxicity screening tests and to assess the strengths and weaknesses of these assays. After an extensive literature search, Battelle has compiled a list of 24 assays, covering seven physiological categories cited by OPTS/EPA. Those categories included nitrogen fixation, photosynthesis, respiration, high-energy phosphate production, growth and cell division, catalysis, and other cellular processes. Brief descriptions of assay methods and tables containing critiques of each assay are presented along with literature references for all of the assays. Assays that are simple, rapid, cost-effective, reproducible, and well-documented are highlighted.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Analyzing/Assessing Bioassay Physiological/Cellular Toxicity	Physiological Toxicity Tests Biological Toxicity Cellular and Subcellular Mechanisms of Toxicity	

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