Methods for Assessing the Effects of Chemicals on Reproductive Functions of Microorganisms

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1. DEFINITION OF REPRODUCTIVE FUNCTION IN MICROORGANISMS

In contrast to the reproductive function and systems of the animal and plant kingdoms, reproduction in the Protista must be considered in significantly different terms, basically because the reproductive unit is directly equated with the complete organism. For most prokaryotic microorganisms reproduction is viewed as growth since increase in cellular size is intimately part of the cell cycle which terminates with cell division. Thus in biosynthetic and organizational terms cell growth is directed towards replication of the genetic material, its controlled separation and its even distribution between two progeny (a process termed binary fission). Indeed for viruses and bacteriophages growth processes have been pared down to a complete minimum and the whole organism may be considered as a reproductive unit capable of its own replication (under appropriate conditions). At the opposite end of the microbial spectrum, particularly within the eukaryotic organisms, such as microalgae and fungi, morphological developments may lead to structural modifications which generate recognizable reproductive structures. Furthermore, among the more complex microorganisms differentiation into 'male' and 'female' types may be recognized: certainly reproductive events requiring the participation of + and − mating types have been described. This situation contrasts with the absence of differentiated forms in lower microorganisms.

In general, therefore, it is usual, convenient and valid to consider reproduction in terms of growth and this is the basic stance that will be adopted in this paper.

2. EFFECTS OF CHEMICALS ON MICROBIAL GROWTH

Chemicals can affect the growth of microorganisms in one of three ways.
2.1 Growth Stimulation

The compound can support or stimulate the growth of a microorganism, acting as a carbon, energy or element resource. By and large these processes are unlikely to be viewed with concern since the compounds involved usually fall within the usual category of compound exploitable for normal metabolic purposes. There may be situations, however, where gross imbalances may generate problems, such as eutrophication processes when the presence of excess inorganic nutrients may trigger the explosive growth of a limited number of species. Such events can have subsequent effects which may influence the reproductive processes of other organisms.

2.2 Growth Depression

The compound can terminate or inhibit, either generally or highly specifically, cellular function(s) which result in the cessation of cell growth and division. That is, the compound can produce a bacteriostatic effect which may be reversed subsequently, permitting normal growth to resume. In the short term these organisms are likely to be metabolically active and able to contribute to any of the processes mediated by the microbe in question: that is, they remain functionally active although unable to grow. In the longer term static organisms will lose viability and the chemical-induced response merges with the next section.

2.3 Lethal Effects

The chemical can induce rapid, lethal effects which result in cell death over a short period of time compared with the effects encountered in section 2.2. Compounds can affect many cellular functions, the precise target reflecting the nature of the compound. For example, the presence of analogues of DNA precursors rapidly affects DNA replication either inhibiting growth, producing DNA free cells or gross morphological changes (e.g. rod-shaped cells becoming filamentous); antibiotics which inhibit the synthesis of cell wall components rapidly produce cells with defective structural components leading to cell membrane rupture and lysis. In general, whatever the cellular target, the observed response normally follows the sequence of cessation of increase in cell number; gross changes in metabolic activity; cell lysis and excretion of intracellular components into the environment.

There are other processes which may be neutral in terms of the growth of microorganisms but which may influence other microbes and other life forms subsequently (for example, bioaccumulation of a compound as a result of trophic structures and food webs and chains). In other systems microbial metabolic activity may result in a transformation which may be potentially more hazardous than the primary compound, a process known as lethal synthesis. Thus, a major conclusion must be that to appreciate fully the potential effect of a compound, it
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is necessary to understand its complete fate in the environment irrespective of its immediate effect upon microbial growth.

The study of the effect of chemicals on microorganisms must be viewed with considerable importance because of their crucial role in establishing and maintaining the operation of the whole biosphere. Effects of chemicals, especially xenobiotic compounds, must be monitored with concern, not only at the local level but at the global level (Kornberg, 1979; Slater, 1981a).

3 METHODS OF ASSESSING THE EFFECT OF CHEMICALS ON MICROBIAL GROWTH

3.1 Standard Growth Procedures

The assessment of an effect on a microorganism is in essence simple and relies on standard microbiological procedures. As the two introductory sections have indicated, the initial measurement of an effect can be gauged in terms of the growth response of a microbial population. Growth has been recognized as the primary method in microbiology with a limited number of basic techniques involving growth in closed (batch), liquid culture; open (continuous-flow), liquid culture; and growth on solid surfaces (e.g. agar-agar added to an appropriate medium contained within a Petri dish).

In the first place the choice of growth medium (i.e. essential nutrients) is normally dictated by the type of organism to be cultured, at least for the more fastidious organisms. It is desirable, but not essential, to use defined media since this facilitates reproducibility, a factor which may be particularly important in comparing data from different laboratories.

3.2 Assessment of Microbial Growth

There are many methods available to measure microbial biomass or microbial cell number concentrations. It is important to recognize that all these methods provide a mean value since microbial populations grown in traditional systems (especially closed culture and as colonies on solidified growth medium) are heterogeneous. Furthermore many environmental factors, including media composition, type of organism, physicochemical factors, substantially influence microbial biomass determinations (Herbert, 1961; Bull, 1974). Indeed it is recognized that one of the attractions of continuous-flow culture systems is that many of these variables may be controlled, leading to more accurate, reproducible estimates of microbial biomass.

3.2.1 Microbial biomass estimates by dry weight determinations

The standard method is to take a known volume of culture (sometimes treating the cells with an agent which terminates growth, such as 1% (w/v) sodium azide
or formalin or gluteraldehyde), centrifuge to precipitate the organisms in a preweighed glass centrifuge tube and dry to constant weight in an oven at 105°C. Accuracy, unless using very large culture volumes, is normally a problem and must be subjected to rigorous statistical control through appropriate replications. Variations on this procedure include filtering through preweighed membrane filters.

3.2.2 Microbial biomass estimates by turbidity determinations

The microbial biomass, under appropriate conditions particularly at lower concentrations, is some function of the amount of light scattered or absorbed by the culture. Many devices, including colorimeters, nephelometers, spectrophotometers, are commercially available to estimate either unabsorbed light or scattered light and have been described in detail elsewhere (Meynell and Meynell, 1964). An important problem with turbidity or absorbance measurements is that cell morphology, a variable parameter depending on the growth conditions, influences the values obtained. With well-characterized systems, the techniques are valuable and have the advantage over dry weight determinations in being rapid, effectively giving instantaneous results.

3.2.3 Microbial biomass estimates by specific cellular component analysis

Many cellular components including total nitrogen, protein, phosphorus, DNA, RNA and others have been assayed, usually by standard colorimetric assays and, using a known percentage composition figure, these values can be used to calculate microbial biomass in terms of dry weight per unit volume. Often the procedures are time consuming but the serious limitation is that microbial macromolecular and elemental composition is not constant and unless the precise variations as a function of, say, organism growth rate, are known, these procedures are unreliable.

3.2.4 Microbial biomass estimates by total carbon analysis

In contrast to specific components mentioned in section 3.2.3, the carbon percentage of organisms is relatively stable for most standard growth media and conditions may be used as a method of calculating biomass. Recent developments have seen the introduction of several reliable, commercially available total organic carbon analysers which are simple to use, require very small samples and provide rapid estimates of the organic carbon content of cells. This ought to be the method of choice with the major disadvantage being the cost of the equipment.

3.2.5 Total cell number estimates

The total number of microbial cells, that is, without distinguishing between
viable and non-viable members of the population, can be estimated by microscopic counts using calibrated counting chambers. A variety of counting chambers are commercially available and enable the number of organisms present in a suspension (normally a growing culture considerably diluted by a known amount) to be counted in a known chamber volume. The procedure is time consuming and requires a high degree of technical accomplishment to obtain accurate and reproducible estimates. Under some circumstances, usually a highly defined organism/growth system, electronic particle counters, such as the Coulter counter, may be used to estimate total cell numbers. This procedure relies on the absence of inert or non-microbial particles in the suspension which is sometimes difficult to achieve. The procedure requires the cells to pass separately through an orifice less than 100 μm in diameter which is frequently difficult to achieve consistently.

3.2.6 Viable cell number estimates

For the majority of microbial experiments the significant parameter is the number of viable cells present within a population since these contribute to the overall metabolic function of the population and to the growth potential of the population. In many instances the viable count agrees closely with the total cell number estimate, especially under optimum growth conditions: that is, there are few non-viable, non-growing organisms in an actively growing population. Indeed, in many instances, the observed discrepancies are probably due to difficulties in estimating reliably the viable count. Viable counts are normally determined by preparing all appropriate dilutions of a growing culture (suitable precautions must be taken to preserve viability at this stage, for example, using buffered diluents, prewarmed diluents, rapid preparation, etc.) and evenly spreading a known volume of a known dilution over the surface of a suitable growth (recovery) medium. This distributes, ideally, single organisms over the growth medium’s surface, suitably spaced. The spread plates are incubated at an appropriate temperature, leading to the development of macroscopic colonies which are assumed to have been derived from a single, viable organism originally spread onto the surface. By calculation the number of colonies value can be used to estimate the number of viable organisms present in the original culture. The procedure is time consuming, requiring at least an overnight incubation, although variations, such as the slide culture technique, can estimate viable cell numbers more rapidly.

3.3 Growth Parameters Capable of Determination

Microorganisms generally are capable of a wide response of overall growth potential, influenced and modified by the environmental conditions. In fact in many instances microbiologists need to be aware of the variable responses made
by apparently identical populations growing under apparently identical growth conditions. This is an attitude which demands appropriate replicative control and statistical analysis. Furthermore it means that searching for and quantifying the effect of a given chemical on the growth processes requires comparison with appropriate control growth systems established in the absence of the stress induced by the chemical.

In conjunction with the basic methods outlined in section 3.2, the following growth parameters can be assessed in relation to treated and untreated growth systems.

3.3.1 The closed culture growth cycle

In closed culture growth systems, microbial populations exhibit a characteristic sequence of growth stages or phases described in detail elsewhere (Bull, 1974; Pirt, 1975; Slater, 1979). Useful, general information may be obtained by determining the effect of a compound on the length of the various growth phases, the size of the maximum population phase culture and other parameters, such as culture viability, cell morphology and/or differentiation, and metabolic capabilities. The last factor, metabolic capabilities, is beyond the scope of this contribution since any cellular capability ranging from an overall process, e.g. the rate of oxygen uptake, to an extremely specific capability, e.g., the activity of a particular enzyme, could be assayed. It is not possible or useful to specify these processes which ought to be selected with regard to the nature of the compound under study. In some circumstances due regard may have to be taken to legislative regulation and control agency requirements. For example, the US Environmental Protection Agency requires, for pesticides, an evaluation of their effect on oxygen consumption and carbon dioxide evolution rates, nitrogen cycle reactions (where appropriate) and measurements of phosphatase or dehydrogenase activities. Variations between the control and treated growth systems as a function of the stage of the closed culture growth cycle might also provide useful information.

3.3.2 The maximum specific growth rate, $\mu_{\text{max}}$

The maximum specific growth rate, $\mu_{\text{max}}$, (units: time$^{-1}$), measures the optimum growth rate for a given organism under a given set of environmental conditions and is the simplest growth parameter which may be deduced from growth kinetic data. The parameter measures the rate of growth under balanced growth conditions which exist during the exponential phase of closed culture growth or during washout from a continuous-flow culture system: the methods of calculation and a detailed discussion of this parameter are given by Bull (1974) and Slater (1979). Differences in the $\mu_{\text{max}}$ values obtained between treated and untreated growth systems provide a useful and quantitative description of the overall effect of a compound on the growth of an organism.
3.3.3 *The saturation constant, $k_s$.*

This parameter measures the affinity an organism has for a particular, growth-limiting substrate and for most nutrients is preferably determined from continuous-flow culture (chemostat) data (Slater, 1979). The influence of a compound on the $k_s$ value obtained may be determined but it must be stated that this requires elaborate, sophisticated chemostat equipment (especially if much credence is to be placed on the numerical values obtained); this information is technically difficult to obtain; and requires an extensive experimental programme.

3.3.4 *The inhibition constant, $k_i$.*

From growth data, especially continuous-flow culture systems, it is possible to determine the $k_i$ value for the chemical under test and to determine the nature of its action, i.e., competitive, non-competitive inhibition, etc. (Bull and Brown, 1979). Again these are likely to require lengthy experimental procedures.

3.3.5 *The minimum inhibitory concentration, MIC value.*

An important and relatively straightforward part of a chemical’s evaluation programme ought to be the determination of its minimum inhibitory concentration (MIC). Normally this may be satisfactorily evaluated in closed culture systems and ought to be tested at an early date in order to provide a set of baseline data in the light of which other experimental programmes (such as those described in sections 3.3.1–3.3.4) may be conducted; there is little point in collecting $\mu_{\text{max}}, k_v, k_i$, etc. data using inappropriate concentrations which may be too low to have any measurable effect or too high to measure useful effects. Furthermore this baseline data is crucial in another context: it enables comparisons to be drawn between microbially harmful concentrations and concentrations known to occur, on average, in the environment. This is particularly significant since there is a tendency to over-react to datum profiles obtained at environmentally inappropriate and inconsequential compound concentrations. There is no point in curbing the use of a particular compound if the measured effect occurs at concentrations unobtainable in nature: regulatory bodies are in danger of justified ridicule through ill-considered decisions!

**4 STRATEGIES FOR THE APPLICATION OF STANDARD METHODS OF CHEMICAL EFFECT EVALUATION**

Section 3 outlined the basic procedures which may be employed to evaluate the effect of a chemical on the growth of a microorganism. In essence these procedures are simple and the significant features of the evaluation programme
lie elsewhere. This section deals with a number of important constraints and principles which need to be taken into account if a satisfactory evaluation is to be achieved.

4.1 Microbial Diversity

It is perhaps an axiom but worth stating in the context of the Workshop that within the microbial world there is a wider spectrum of physiological types and metabolic capabilities than in any other group of organisms and, indeed, in the total of other organisms. This microbial heterogeneity, in terms of energy metabolism and anabolic and catabolic potential, raises a serious problem in terms of evaluating the effect of a particular chemical. Stated simply, great care must be taken in choosing a test organism since that organism must possess an appropriate cellular function as the target for the chemical. For example, evaluating the effect of a compound which specifically interferes with nitrogen-fixation processes would be pointless using a non-nitrogen-fixing organism. Furthermore the evaluation programme would similarly be invalidated if a nitrogen-fixing organism was grown under conditions where the mechanism was not elaborated (e.g. a filamentous cyanobacterium grown in the presence of ammonium ions does not differentiate to produce heterocysts and so does not fix nitrogen). Similarly, the effect of a compound which selectively impairs substrate level phosphorylation would be substantially less marked using a test organism grown aerobically compared with the same organism grown anaerobically.

Within compounds with known targets or suspected targets, the selective use of appropriate test organisms is justified. However, in the majority of instances, particularly for xenobiotic compounds, where their mode of action is not understood, then probably the only course of action would be to screen the compound against a range of metabolic types, certainly including those with major capabilities of importance to the natural environment, such as nitrogen fixation, phosphous mineralization, etc. Clearly screening programmes are labour and time intensive but, in the absence of detailed knowledge of the compounds effect, ought to be part of an evaluation programme.

4.2 Microbial Communities

Most microbiologists are imbued with an experimental approach which depends on the use of axenic cultures, i.e. the growth of a single species of microbes. This is clearly a valuable part of an evaluation programme to test effects on a selected range of known organisms with characteristic metabolisms. However, in virtually every known natural habitat, mixtures of microorganisms coexist, in some cases resulting in the formation of stable, interacting communities (Slater, 1978, 1981b). It is becoming clear that many types of interaction, ranging from the genetic to the physiological level, occur which may
substantially modify the overall metabolic potential in a way which is not
exemplified by any of the pure cultures alone. It is conceivable, but thus far
untested experimentally, that microbial communities could transform a non-
toxic compound into a toxic compound. This is an area which requires much
more experimental work. On the other hand, and in fact a more likely sequence of
events, is that a particular problem compound will be transformed (normally
completely mineralized) more readily and more rapidly by a mixture of
microorganisms acting synergistically (Bull, 1980; Slater and Godwin, 1980).
That is, the long-term persistence of a compound in the environment may be
considerably shorter than would be deduced from pure culture studies alone. In
situations where there is a balance between the need to use a given compound and
its deleterious effects, then the more rapid its degradation, the more desirable.
Microbiologists in general seem reluctant to consider mixed culture studies but
unless they are encouraged to do so, they will continue to furnish data which in
many cases bears little relation to the processes occurring in nature.

4.3 Environmental Heterogeneity

A comparatively unexplored area, certainly in terms of chemical safety
evaluation, is that of environmental variation and heterogeneity. Most simple
experimental systems, such as a closed culture, depend on the homogeneous
dispersion of microorganisms in a culture medium to produce a homogeneous
suspension. These are conditions which rarely occur in nature: various types of
surfaces and interphases occur (Marshall, 1980) and, indeed, most microbial
activity occurs at these regions. Here physicochemical factors lead, for example,
to major nutrient gradients which in turn affect the rate at which organisms grow.
By and large heterogeneous growth systems have not been adequately explored
in the present context.

4.4 Microbial Adaptation

In many instances test procedures fail to allow for adaptive processes,
particularly those which may occur at the genetic level (as opposed to phenotypic
adaptation or expression of a particular function). In the long term these events
may be important in alleviating the effects of chemicals by the selection of
mutants with desired resistance characteristics or, more importantly, by the
selection of strains able to degrade the test compound. Evaluation programs
ought to include some estimations of the likelihood of these events occurring but
it is, of course, difficult and often a long-term programme. Nevertheless, as has
been observed in other contexts, ultimately the immediate effect of the chemical
may not be such a problem as was anticipated in the unadapted population
(Slater, 1978).
4.5 Evaluation in situ and Microcosms

As indicated elsewhere in this section, one of the major experimental problems is that of relating laboratory-based experimentation—often with highly defined, simple growth systems—to the likely behaviour of the compound in natural habitats. At some stage field studies need to be undertaken to make the necessary comparisons. An intermediate stage, however, is the use of more complex growth systems, normally termed microcosms which attempt to establish a great ecosystem complexity within a laboratory-based system (Matsumura, 1979; Bull, 1980).

4.6 Microbial Systems as Models for Testing Effects of Chemicals

In much the same way as microbial systems have been analysed as convenient life forms from which the basic fundamentals of living systems may be deduced, microbial systems may, in part, serve as convenient models to test the effect of chemicals before testing on animals and plants. The relative simplicity of microorganisms, the ease of culturing and the ability to produce large quantities of material for analysis, enables them to be used in useful preliminary tests to determine likely cellular targets for the compound in question. For example, results may indicate an influence on DNA synthesis or protein synthesis inhibitor, and these results may indicate what cellular functions need to be examined in higher organisms. Clearly extrapolations of this kind need to be attempted cautiously and with due regard to the substantial differences exhibited in higher organisms. This strategy has, for example, been used as the basis of the Ames test for determining those compounds which tend to have carcinogenic effects.

5 CONCLUSIONS

The major conclusion which it is hoped is clear from the preceding test is that the basic methodologies already exist for the analysis of the effect of chemicals on microbial growth. To this author, however, there are some serious limitations to the application of these methods, especially in the range and level of complexity of the assessment tests undertaken. Much more thought needs to be given to the experimental protocols developed, taking into account the factors described in section 4.

6 REFERENCES

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