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A Rapid-Cell Culture Assay of Water Quality

S. R. VanDoren, M. S. Hall, L. B. Frazier, and F. R. Leach

Department of Biochemistry, Oklahoma State University, Stillwater, OK 74078

Assessment of the quality of water obtained from either natural sources or of water returned to the environment after use is required to establish that no pollutants are present which could be injurious to any of the various living things that would utilize the water. Bioassays offer a means of establishing water quality. Aquatic organisms of various types are routinely used for bioassays (Cairns and van der Schalie 1980) and the use of such organisms allows estimation of the effect of bioaccumulation (Baughman and Paris 1981). Simpler systems involving less than the whole organism might add utility to the assay provided that enough biochemical reactions are still required to yield the measured parameter. Cells growing in culture provide a useful model for which many experimental methods have been published (Kruse and Patterson 1973; Jakoby and Pastan 1979).

This laboratory has used cells growing in suspension culture to detect and measure toxicity in oil refinery effluents (Richardson et al. 1977). We have also developed a procedure for growing cells on glass fiber filters which yields replicate cultures for the determination of biosynthetic activity (Stadnicki and Leach 1978). The use of cells in culture are advantageous because their growth depends on the fidelity of DNA replication and both RNA and protein synthesis. About 10^5 cells are involved in the assay which gives a sufficient population of cells for a great statistical advantage over fish or Daphnia assays, which may involve only 20 individuals.

In this paper we report results of our experiments to develop a cell culture screen based on thymidine incorporation during a four-hour incubation to determine water quality of environmental samples. A rapid screening type of test was desirable to establish which samples would require further testing by more expensive and time-consuming procedures. The assay described can be completed in one day if the precipitate is dissolved in warm Soluene and left to be counted overnight.

MATERIALS AND METHODS

HeLa cells (obtained as CCL 17 KB cells but now identified as a

HeLa contaminant) were purchased from the American Type Culture Collection Repository. Cytodex beads were from Pharmacia. Soluene and Insta-Gel were purchased from Packard Instruments. [^{3}H]-Thymidine was obtained from Swartz-Mann. Gibco was the source of the Medium 199 concentrate and calf serum was obtained from Microbiological Associates. Hydroxyurea was from the Aldrich Chemical Company and m-cresol from Baker Chemical. The oil refinery effluents were obtained from samples provided to the OSU Water Quality Laboratory and given as numbered samples whose ultimate source was unknown to us. The Oklahoma State Department of Health provided the Tar Creek water samples.

HeLa cells were grown on Medium 199 + 10% calf serum in milk dilution bottles. The cells were harvested by scraping the bottle with rubber-tipped policemen. The mixture of cells and medium was centrifuged at 220 X g for 10 min. The supernatant solution was poured off and replaced with 2 ml of Medium 199 without calf serum. The cells were suspended by mixing thoroughly with a pipet and then counted with a hemocytometer. These cell suspensions were then diluted with Medium 199 + 10% calf serum and the desired quantities were removed and centrifuged again. The supernatant solution was poured off and replaced with fresh Medium 199 + 10% calf serum. Sterile 125-ml Erlenmeyer flasks treated with a silicone were used for growth of cells on the microcarriers. The Cytodex microcarrier beads were stored at a concentration of 1 g/50 ml in sterile phosphate-buffered saline. Immediately before use, the microcarriers were washed three times with Medium 199 + 10% calf serum and left in a volume of medium equivalent to that of the initial volume of phosphate buffered saline. To each flask was added 0.04 ml of the suspension of microcarriers per ml of final volume to be in the flask. Each flask was inoculated with 5 X 10^4 cells per ml of final volume, and the volume in each flask was brought to 5 ml with fresh medium. The flasks were incubated without shaking at 37° for two h to allow attachment of the cells to the microcarriers. The volume was then adjusted to 25 ml. The flasks were incubated on a New Brunswick G-10 Gyrotory shaker at 50 cycles per min in a 37° room for 16-24 h.

The fluid containing the cells was transferred to a silicone-treated glass tube. The beads with the attached cells were allowed to settle for 5 min. The supernatant solution was carefully removed and discarded. A volume of fresh 2 X Medium 199 + 10% calf serum equivalent to one-half the final volume was added. Then 0.1 μCi of [^{3}H]-thymidine was added per ml of final culture volume, and the liquids were completely mixed. Then 1 ml of the culture containing the labeled thymidine was added to each sample vial. The preparation of the sample vials is described below.

Sterile glass-distilled-water (if required) was mixed with the Millipore-filtered environmental sample to yield concentrations of 0, 25, 50, and 100% of environmental samples. Wheaton glass liquid scintillation vials, 15 X 50 mm

with a nominal volume of 6 ml, were prepared and each supplemented with 1 ml of the appropriate concentration of sample and 1 ml of culture already mentioned. At least three replicates were done for each concentration. The vials were capped and placed on a rack which was inclined 45° on a shaker operating at 50 cycles per min in a 37° room. The cultures were incubated 4 h.

Each culture was diluted with 4 ml of water and the vials were centrifuged for 5 min at 450 X g. All but one ml of the diluted medium was removed by suction from each vial. Five ml of 10% trichloroacetic acid was added. The contents of the vials were mixed by vortexing and then centrifuged 10 min at 450 X g. All but one-half ml of the supernatant solution was removed by suction. The trichloroacetic acid treatment was repeated and as much as possible of the supernatant fluid was removed. One-half ml of Soluene 350 was added. If the Soluene was cold the precipitates were incubated overnight, but if the Soluene was warm the precipitate would dissolve in an hour. The use of Soluene increased the observed counts by 2.5-fold.

Five ml of Instagel scintillation cocktail was added to each vial and the contents were mixed by vortex treatment. The radioactivity in the vials was determined using a Packard Prias 400 CL/D liquid scintillation spectrometer. The counts were taken for 10 min after the vials had been in the scintillation counter for at least 5 h. The mean sample incorporation was calculated as a per cent of the mean control incorporation. The sample standard deviation was calculated for each mean incorporation.

Table 1 summarizes the various parameters tested in developing this procedure.

RESULTS AND DISCUSSION

In our previous studies we found a greater incorporation of thymidine than of uridine or tyrosine. In data not shown these observations were confirmed using microcarrier beads. We used hydroxyurea, a known DNA synthesis inhibitor, to show that the assay would detect the inhibition of thymidine incorporation during the 4h-incubation period. Figure 1.A shows the effect of increasing hydroxyurea concentrations on thymidine incorporation. This curve is similar to that observed with cells growing on glass fiber filters with a longer incubation period (Stadnicki and Leach 1978), but of course the amount incorporated was reduced using the shorter incubation time.

A common organic compound found in polluted waters, m-cresol, was also tested. Figure1.B shows that increasing concentrations of m-cresol produced increasing inhibition of thymidine incorporation. These observations are consistent with the inhibitory effect of m-cresol when tested with a longer incubation period using suspension cultures (Richardson et al.

1977). Figure 1.C shows the $ZnSO_4$ inhibition.

These observations show that a 4-h incubation period with tritiated thymidine is sufficient to detect inhibition of radioisotope incorporation.

Table 1. Summary of the development of the method

Parameter Tested	Range or test	Result
Cell type	HeLa vs L	HeLa grew better when a lower number of cells were used
Growth volume	Culture vessel	12-25 ml no effect; 25 ml decreased incorporation
Pulse time		4 h gave optimum sensitivity, minimum variability, and was consistent with one-day exper.
Experimental vessel	Test tube vs scintillation vial	Slightly greater incorporation in vial; easier to use
Microcarrier beads	Cytodex vs Biocarrier	Cytodex 3-4 X incorporation
Growth support	Microcarrier vs glass fiber filters	Incorporation with beads is 5 X greater than with filters
Amount of isotope		Proportional to concn. from 0.05 to 0.2 μCi/ml
Amount of beads	0.4-1.2 mg/ml	No variation
Cell number		Between 0.4 and 0.8 X 10^5/ml proportional to cell number Optimum 4 cpm/cell # with 8 X 10^4/ml

We will illustrate the use of the assay with two types of environmental samples. Oil refinery effluents obtained from the OSU Water Quality Laboratory were from various holding ponds. The compounds present in the samples vary greatly (both in their nature and concentration) and give a range of toxicities. Table 2 shows the inhibitions produced by typical oil refinery effluent samples. Figure 1.D shows typical inhibition results.

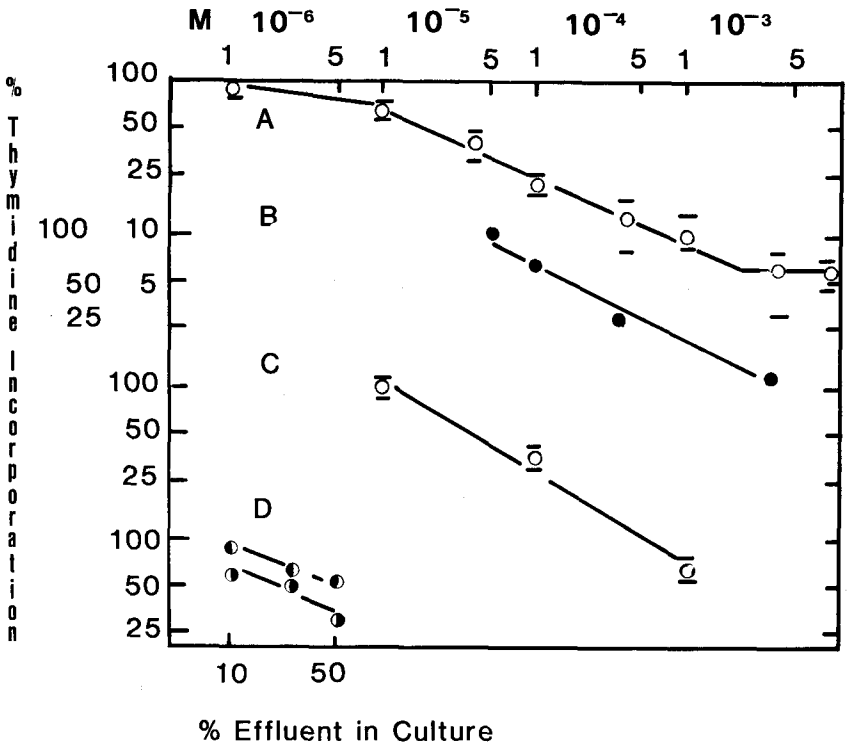

Figure 1. The measurement of toxicity by following thymidine incorporation.
Part A. Inhibition of thymidine incorporation by hydroxyurea. HeLa cells were exposed to the indicated concentrations of hydroxyurea during a 4-h incubation period. The values shown are means of four determinations and the standard deviation is shown by the lines above and below each point.
Part B. Inhibition of thymidine incorporation by m-cresol. HeLa cells were exposed to the indicated concentrations of m-cresol during a 4-h incubation. The values shown are means of four determinations.
Part C. Inhibition of thymidine incorporation by $ZnSO_4$. The indicated concentrations of $ZnSO_4$ were used.
Part D. Inhibition of thymidine incorporation by two oil refinery effluent samples. The effect of a less inhibitory sample, ◐, and a more inhibitory sample, ◑, on thymidine incorporation are shown.

The second series of samples were from the Tar Creek area of Oklahoma. Tar Creek is the principal drainage system of the Picher field which was one of the most productive lead-zinc mining districts in the United States. While mining operations were in progress, the ground water was removed using large-capacity pumps to prevent water intrusion into the mine shafts and tunnels. This produced a cone of depression until the mining operations were suspended. Then ground water infiltration and direct surface

water inflow recharged the Boone formation. Many of the oxidation products were solubilized and thus contaminated the water. Tar Creek has high concentrations of cadmium, iron, lead, and zinc, a low pH, decreased dissolved oxygen, and an increased dissolved solids concentration. Tar Creek ranked first on the list of 114 sites that EPA listed for implementation of the Comprehensive Environmental Response, Compensation and Liability Act. Site # 20 is on Tar Creek near Miami, OK, site # 22 is just before the confluence of Tar Creek and the Neosho River, site # 24 is on Spring River, and site # 26 is the inlet of Tar Creek drainage into the Lake of The Cherokees. The results of our testing shown in Table 2 are consistent with the extent of travel of the acid mine water. Only the sample from site # 20 was markedly inhibitory. This sample contains iron and zinc.

Table 2. Results with environmental samples

A. Oil Refinery Effluents

Sample #	% of Control TdR Incorporation
1	55
2	81
3	49
4	80
5	32
6	74
7	72
8	47

B. Tar Creek Sample

Sample site #	Sample Concentration %	% of Control TdR Incorporation
20	25	10.2
22	50	98.7
24	50	101.8
26	50	108.0

During the course of the experiments we noted that with the oil refinery effluent samples there was decreased toxicity in subsequent assays (29-30%). The samples were not collected aseptically and were not filtered until just before use in the cell culture assay system.

The growth of cells on microcarrier beads provides the advantages of suspension cultures for cells that do not grow in suspension and yields a system where a number of replicate cultures can be used. The microcarrier beads are kept suspended by gentle shaking and the mixing that occurs gives a more effective utilization of the medium components. Microcarrier cultures (Mered et al. 1980) give a superior yield of cells per milliliter of tissue culture medium. Microcarrier cultures offer the advantage that they can be scaled up or down with respect to the volumes involved.

This paper describes the development of a cell culture assay using cells growing on microcarrier beads and a 4-h thymidine incorporation as a screen for water toxicity. The advantages of the system are: 1) the use of a small volume of water in the test, 2) the ability to have a large number of replicate cultures, 3) the ability to do the test and have the final result within a 24-h period, 4) the use of a large number of cells which eliminates the complications produced by using a few organisms in a bioassay, and 5) the capability of using other biosynthetic processes to assess toxicity. Just as in any other bioassay there are limits of the specificity of the organism or cells used (that is, not being sensitive to a particular inhibitor) and the constraints of the time of the assay (chronic, acute, cumulative, lethal, sublethal). The cell culture assay described was developed to serve as a rapid screen to indicate that a particular water sample requires detailed investigation using a battery of tests.

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