# Agar Diffusion Method for Toxicity Screening of Plastics on Cultured Cell Monolayers

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**In a previous paper, a tissue culture method (fluid-medium) was used to screen the toxicity of plastic materials to be used in medical practice. Even though this method was found to be very acceptable for most plastic materials, some difficulties were encountered when plastics having low densities or plastics having odd shapes or forms were tested. For this reason an agar-diffusion method was adapted which could be used for the testing of any type of plastic as well as for the testing of solid and liquid extracts.**

A PREVIOUS paper from this laboratory described the use of a tissue culture method for screening potential toxicity of plastics to be used in medical practice (1). The method involved placing plastic test samples in contact with monolayers of strain L-929 mouse fibroblasts (L-cells) in Eagle's medium (2) and then observing the surrounding cells microscopically for toxic manifestations.[1]

Using the technique mentioned above, however, presented certain problems with plastic samples of low density or samples of plastics having odd shapes or forms. These samples would tend to float or move about in the liquid medium, and even though accurate results could be obtained, much more time and care had to be given, thereby reducing the efficiency of a rapid screening program. For this reason, it was felt that another tissue culture technique should be adapted which could be used with any plastic material without severe loss of sensitivity.

Preliminary experiments established the usefulness of a tissue culture technique employing a monolayer of cells overlayed with a solid phase of an agar mixture originally developed by Dulbecco (3) in 1952 and since adapted by other investigators (4–6) for toxicity measurements.[2] To fit the needs of our screening program on plastics, certain modifications were made, and both replicating (L-cells) and nonreplicating (chick embryo cells) systems were used. It was felt that these two systems could add greater versatility to the tests while increasing the accuracy of the results. The agar diffusion method could also be employed for liquid and solid extracts from plastics if this were so desired. This paper, however, reports only the studies conducted on plastic samples.

## EXPERIMENTAL

**Materials and Supplies.**—Plastic samples (100 different samples). These samples were previously examined by the fluid–medium tissue culture method (1) and by an intramuscular implantation technique in rabbits (7). Strain L 929 mouse cells (L-cells), chick embryo cells (10 days old), and calf serum (Microbiological Associates, Inc., Bethesda, Md.).

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[1] Referred to as the "fluid-medium method."

[2] Referred to as the "agar-diffusion method."

**Phosphate Buffered Saline Solution (PBS I).**—Sodium chloride, 80.0 Gm.; potassium chloride, 2.0 Gm.; sodium phosphate, dibasic, 11.5 Gm.; potassium phosphate, monobasic, 2.5 Gm.; and deionized water, up to 1000.0 ml.

**Phosphate Buffered Saline Solution (PBS II).**—PBS I, 100.0 ml.; streptomycin–phenol red concentrate, 10.0 ml.; magnesium chloride, as 20% solution, 0.5 ml.; calcium chloride, as 11% solution, 1.0 ml.; and deionized water, up to 1000.0 ml.

*Note:* Just before use, 100,000 units of penicillin G potassium was added to each 1000 ml. of solution.

**Streptomycin–Phenol Red Concentrate.**—Streptomycin sulfate (Pfizer & Co., New York, N. Y.), 5.0 Gm.; phenol red U.S.P. (Allied Chemical Co., New York, N. Y.), 0.5 Gm.; and deionized water, up to 1000.0 ml.

**Eagle's Medium (2).**

**Agar.**[3]

**Neutral Red Solution, 0.01%.**

**Preparation of Tissue Culture Plates.**—Monolayers of chick embryo cells were prepared on 100-mm. Petri dishes as described by Dulbecco. Monolayers of L-cells were prepared by pipeting a volume of cells (approximately $2.4 \times 10^6$ cells) in 10.0 ml. of Eagle's medium (2) into 100-mm. Petri dishes. Both types of cultures were then incubated 48 hr. at 37° in a carbon dioxide (5%) air atmosphere.[4] During the incubation period, full monolayers of the cells formed on the bottom surface of the plates.

All culture dishes were removed from the incubator and the fluid media removed by aspiration. The cell layers were washed once with PBS II supplemented with 1% calf serum, using 5.0 ml. per plate. This liquid was then removed and 10 ml. of Eagle's medium containing 1% agar and 1% calf serum was pipeted into each culture dish.[5] The agar overlay was allowed to solidify at room temperature on a level surface. Each culture was then stained in the following manner.

Ten milliliters of neutral red solution (a vital stain) was gently pipeted onto the center of the overlay surface and the plate rotated for equal distribution of the stain. The stain was allowed to remain on the plates for 15 min. and was then removed by aspiration. The plates were then inverted until ready for use.

**Testing Method.**—Plastic sheets or films were cut into square sections of approximately 2 to 4 mm. in size. Tubings were cut into cylindrical

[3] Marketed as Bacto-Agar by Difco Laboratories, Detroit Mich.

[4] Air–$CO_2$ mixture maintained cultures at 7.0–7.2 pH.

[5] Temperature of liquid medium was approximately 47°.

sections of approximately 2 mm. in length. Two samples of the same plastic were implanted on a culture plate. A positive control plastic (known toxic plastic) and a negative control plastic (known nontoxic plastic) were also implanted on the same plate. Two plates of cells with no implants were run with each set of the test plates as further controls. All materials implanted were handled with sterile forceps (alcohol dipped and flamed) and pressed gently and firmly onto the medium.

After implantation, the plates were inverted and placed into the incubator for 24 hr. The plates were then removed and placed on a surface having a white background. Nontoxic plastic test samples, negative control plastic samples, and control plates (no plastics) were distinguished by the even pink color of the cell monolayer. Toxic plastic samples as well as positive control plastics were characterized by relatively clear colorless zones of dead cells around the samples, while the area of the monolayer unaffected by the samples remained pink. It was possible in many cases to observe a toxic or positive response in much less than 24 hr. Zones of dead cells were sometimes observable within 8 hr., and the progressive widening of such zones could be noted.

## RESULTS AND DISCUSSION

Table I summarizes the results of the study for the 100 samples of plastic tested. As may be seen from the table, these results are compared to those results obtained by the fluid-medium method reported in a previous paper (1). Of the 100 samples tested by the agar-diffusion method reported here, ten samples gave conflicting results as compared to the fluid medium method. For example, by the fluid-medium method the ten samples (X-22, X-24, X-46, X-47, X-70, X-196, X-197-A, X-197-B, X-197-H, and X-197-K) showed positive reactions, while by the agar diffusion method for either the chick embryo cells or L-cells the results were negative. Several factors may have helped to contribute to these inconsistent results, perhaps the main factor being the thickness of the agar overlay which could absorb a portion of the toxic component, thereby reducing the concentration of component reaching the cells. In several further experiments the sensitivity of the agar overlay method was improved by reducing the amount of the overlay from 10 to 5 ml., but this reduced potential longevity of the underlying cell layer.

In four cases, X-22, X-46, X-196, and X-197-K, there were anomalous test results. As may be seen from the table, all of the test results in chick cells and L-cells by the agar overlay method were consistent except for those mentioned which demonstrated toxicity to L-cells and nontoxicity to chick embryo cells. This inconsistency in results may be attributed to the greater sensitivity of the L-cells as compared to the chick cells to the agents in the plastic causing the toxic effects.

Even though not reported here, liquid extracts from plastics sorbed onto sterile paper assay disks (12.7 mm.) and extracts as powders have been evaluated for toxicity by the method described here.

Results of the study reported in this paper as well as further experience with the agar-overlay method demonstrates the feasibility of using this technique for rapid screening of plastic materials with considerable ease and at a relatively low cost. This method in conjunction with other biological testing methods (7) should serve as a rational index to accept or reject a plastic material to be used in medical practice. The agar-diffusion method has the added advantage that liquid and solid extracts may also be tested for toxicity.

## SUMMARY

In order to be able to test all types of plastic samples in this laboratory for the toxicity of plastics to be used in medical practice, an adaption of the Dulbecco agar-diffusion method was used to evaluate 100 plastic samples, previously tested by a fluid medium method using L-cells and by an intramuscular implantation technique in rabbits. Tox-

TABLE I.—COMPARISON OF TEST RESULTS FROM FLUID MEDIUM L-CELLS AND AGAR OVERLAY METHOD FOR 100 PLASTIC SAMPLES

| Test Plastic[a] | Fluid Medium L-Cells | Agar Overlay Method L-Cells | Agar Overlay Method CE Cells |
|---|---|---|---|
| X-1, X-20, X-23, X-26, X-33, X-35A X-39, X-42, X-43, X-44, X-50, X-52 X-60, X-62, X-68, X-69, X-72, X-73 X-83, X-148, X-149, X-154, X-158 X-159, X-169, X-175, X-177, X-178D X-179A, X-182A, X-182B, X-182C X-182D, X-182G, X-182I, X-182J X-185A, X-185B, X-187A, X-187B X-187C, X-194A, X-198A, X-198B X-198C, X-198D, X-198E, X-200A X-200C, X-200D | − | − | − |
| X-2A, X-4, X-6, X-8, X-17, X-18, X-19 X-21, X-25, X-34, X-36, X-37, X-38 X-40, X-45, X-48, X-49, X-53, X-54 X-55, X-61, X-63, X-64, X-65, X-67 X-71, X-74, X-113, X-140, X-145 X-150, X-152, X-168, X-182E, X-182H X-195, X-197G, X-197L, X-197M, X-197O | + | + | + |
| X-24, X-47, X-70, X-197A, X-197B X-197H | + | − | − |
| X-22, X-46, X-196, X-197K | + | + | − |

[a] Code numbers given to each of the plastic samples tested.

icity was revealed through use of a vital staining technique to systems of replicating (L-cells) and nonreplicating (chick embryo cells) types.

Results of the tests demonstrated the usefulness of the techniques for rapid screening of all types of plastics for toxicity and further opened up an avenue for testing of liquid and solid extracts from plastic materials.

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# Gas Chromatographic Analysis of Phenacetin and Probable Contaminants—Acetanilid, *p*-Chloroacetanilid, and *p*-Phenetidin

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**Acetanilid, *p*-chloroacetanilid, and *p*-phenetidin are likely to be present as impurities in phenacetin. U.S.P. XVII describes qualitative tests for acetanilid and *p*-phenetidin and a semiquantitative paper chromatographic limit test for *p*-chloroacetanilid. A gas chromatographic procedure has been developed for the quantitative determination of these three impurities and of the phenacetin content of bulk material. The procedure utilizes Epon 1001 as the stationary phase on Chromosorb G in conjunction with a dual column instrument and a flame ionization detector. The proposed method is rapid and involves only a solvent extraction of the sample and no chemical modification prior to actual analysis. Impurities present in the raw materials in quantities as small as 10 p.p.m. can be estimated. If present in a proportion greater than 50 p.p.m., they may be determined quantitatively.**

DURING the past few years, reports of toxicities associated with phenacetin have appeared in the medical literature. Some of the toxic symptoms have been attributed to impurities introduced during synthesis of the compound. Three impurities likely to be present are acetanilid, *p*-chloroacetanilid, and *p*-phenetidin. Gad (1) has reported that *p*-chloroacetanilid causes methaemoglobinemia. Hald (2) reported cyanosis in patients who had taken phenacetin which was subsequently shown to contain 18% *p*-chloroacetanilid. The 1958 edition of the "British Pharmacopoeia" allowed 0.17% *p*-chloroacetanilid in phenacetin, whereas the 1963 edition lowered this limit to 0.11%. This compendium also has a qualitative test for *p*-phenetidin. The sixteenth revision of the "United States Pharmacopeia" has qualitative tests for acetanilid only. The seventeenth revision has qualitative tests for *p*-phenetidin and acetanilid and allows a maximum of 0.03% of *p*-chloroacetanilid.

The B. P. test for *p*-chloroacetanilid is based on the procedure by Hald (3). This involves cleavage of the aromatic chloride bond with Raney nickel catalyst in the presence of sodium hydroxide, followed by visual estimation of the opalescence produced by the addition of silver nitrate solution. In the U.S.P. test, the *p*-chloroacetanilid is separated by reversed phase paper chromatography and the fluorescence produced on irradiation with ultraviolet light is estimated visually. These procedures are only semiquantitative. Jones and Page (4) have described a polarographic method for the determination of *p*-chloroacetanilid and have shown data for phenacetin which contained 0.1 to 0.34% of the impurity. Recently, Crummett *et al.* (5) reported a very sensitive procedure for *p*-chloroacetanilid. This involves hydrolysis with hydrobromic acid to *p*-chloroaniline and spectrophotometric determination following extraction from a basic medium. This procedure is subject to interference from acetanilid.

Both the B.P. and U.S.P. tests for *p*-phenetidin involve a color reaction with iodine T.S. The authors have found this reaction to be sensitive to concentrations as low as 0.003%. The U.S.P. test for acetanilid involves bromination of a saturated aqueous solution of phenacetin and visual observation of the turbidity due to the bromo derivative of any acetanilid present. This test was found to be sensitive only to concentrations greater than 1.2%. In addition, it can be misleading due to precipitation of some of the phenacetin from the saturated solution.

The purpose of this investigation was to develop a sensitive procedure for the quantitative determination of phenacetin and probable contaminants: acetanilid, *p*-chloroacetanilid, and *p*-phenetidin by gas-liquid chromatography. Such a procedure might aid toxicity evaluations especially in view of the recent FDA label warning required for phenacetin preparations.

## EXPERIMENTAL

**Apparatus and Materials.**—F and M model 810 dual column gas chromatograph with a flame ionization detector and equipped with a Minneapolis Honeywell recorder and disk integrator was used.

The phenacetin (Mallinckrodt Chemical Works) used in the preparation of the calibration curve and synthetic mixtures was recrystallized and shown

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