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# Toxicity Screening of Biodegradable Polymers. II. Evaluation of Cell Culture Test with Medium Extract

**M.-H. Dang,[1,2] F. Birchler,[1] and E. Wintermantel[1]**

Cell culture testing with material extracts was applied to toxicity screening of some commercial degradable plastics: a plasticized cellulose acetate, an aliphatic polyester (Bionolle), polyhydroxybutyrate-co-hydroxyvalerate (Biopol), and polycaprolactone (TONE polymer). Cell culture medium with serum was used as extraction medium. Methods for the determination of morphology and viability of cells cultured in the extract were investigated. Phase-contrast light microscopy of cells, enhanced by neutral red staining, provides high-contrast images for qualitative evaluation of cell morphology and lysis. Compared to the determination of protein using the Bradford method and of neutral red uptake, the determination of dehydrogenase activity using 3-[4,5-dimethylthiazol-2-yl]-2,5-diephenyl-tetrazolium bromide (MTT) is more sensitive and accurate. The relative MTT activity of cells cultured in fresh extracts indicate that TONE polymer (all shapes) and Bionolle (test bars and films) are comparable to materials currently used in the food industry (polyethylene terephthalate, atactic and isotactic polystyrene) with no toxic effects on cells.

**KEY WORDS:** Toxicity screening; biodegradable polymers; cell cultures; test with extract.

## INTRODUCTION

Based on the standard, *in vitro* tests for toxicity on living cells (cytotoxicity) of medical devices and materials [1–3], cell culture tests with the extract of polymers were chosen as the screening method for potential toxicity of biodegradable polymers [4]. This method is advantageous because it can easily be performed and provides reproducible results. In addition, the effects of different materials on cells can be compared with each other.

In this work, the cell culture test with extracts was applied to commercially available, degradable plastics: a plasticized cellulose acetate, an aliphatic polyester (Bionolle), polyhydroxybutyrate-co-hydroxyvalerate-(Biopol), and polycaprolactone (TONE polymer). Methods for qualitative and quantitative evaluation of cell morphology, activity, and growth in the test with extracts were studied to propose a simple and clearly defined test protocol. Based on the effect of their extracts on cells, test materials for the same shape, i.e., test bar, film, or granules, were compared with the standard reference materials for cytotoxicity tests—polyethylene (PE) and organic tin-stabilized polyvinylchloride (PVC†). In addition, materials currently used in the food industry—polyethylene terephthalate (PET), atactic polystyrene (a-PS), and isotactic polystyrene (i-PS)—were incorporated into the cell culture experiments. They served as additional references for the evaluation of materials in daily life.

## EXPERIMENTAL

### Materials

Four commercial biodegradable plastics—a plasticized cellulose acetate, an aliphatic polyester (Bionolle), polyhydroxybutyrate-co-hydroxyvalerate (Biopol), and polycaprolactone (TONE polymer) were studied (Table I). Granules were provided by their man-

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**Table I.** Polymeric Test and Reference Materials

| Sample | Sample shape | Abbr. | Producer |
|---|---|---|---|
| Cellulose acetate (containing plasticizer) | Bars<br>Film<br>Granules (PM 15373) | CA | Eastman Chemical |
| Bionolle | Bars<br>Film<br>Granules (3020) | Bion | Showa High-Polymer Co., Ltd. |
| Biopol (containing plasticizer) | Bars (D300G; 6% HV)<br>Film (D611, 12% HV)<br>Granules (D300G) | PHBV | Zeneca |
| Tone polymer | Bars<br>Film<br>Granules (P787) | PCL | Union Carbide |
| Polyethylene (Lupolen) | Bars<br>Film<br>Granules (3020D) | PE | BASF |
| Organic tin-stabilized PVC | Sheet (stabilized with Irgastab T41M) | PVC† | Kunststoffinstitut, Darmstradt, Germany |
| Polyethylene terephthalate | Mineral water bottle | PET | |
| Isotactic polystyrene | Disposable plate | i-PS | |
| Atactic polystyrene | Disposable cup | a-PS | |

ufacturers. The charge numbers are given in parentheses. Films and test bars, if not indicated otherwise, were processed at the Fraunhofer Institute for Food Packing, Munich, Germany. Noncytotoxic polyethylene (PE) and cytotoxic organic tin-stabilized polyvinylchloride (PVC†) served as standard reference materials for the cell culture tests. A food-grade polyethylene terephthalate bottle (PET) for mineral water, a disposable atactic polystyrene cup (a-PS), and an isotactic polystyrene plate (i-PS) were bought at the supermarket. They served as standards for materials in the food industry.

## Cell Culture Test with Medium Extracts

### *Cell Cultures*

DMEM (Dulbecco's modified Eagle medium; GIBCO 41965-039), supplemented with 5% FBS (fetal bovine serum: GIBCO 10106-078) and 0.2% gentamicin (GIBCO 15710-031), was used as cell culture medium for fibroblasts. Mouse fibroblasts (cell line 3T3, passage number between 40 and 50) were incubated in the cell culture medium in an atmosphere of 5% $CO_2$ and 95% humidity at 37°C until a confluent monolayer was formed. The cell monolayer was then dissociated with trypsin–EDTA solution (GIBCO 45300-019) and resuspended in the cell culture medium. The resulting single-cell suspension was subsequently mixed as thoroughly as possible before seeding into the wells of microtiter plates.

### *Medium Extracts*

Prior to the extraction in cell culture medium with serum, small pieces of test and reference materials were sterilized with $\gamma$ irradiation at a dose of 15 kGy. The ratio of the sample surface to the volume of the extraction medium was kept constant between 0.5 and 6 $cm^2$/ml (Table II). If the surface area was indeterminate, as for granules, 0.1 g/ml was used [3].

After shaking at 80 rpm for 3 days at 37°C followed by a settling period in a sterile hood for 1 h, the extract was separated from the sample by carefully

**Table II.** Ratio of the Sample to the Cell Culture Medium Used for the Extraction

| Sample shape | Ratio of sample surface (or weight) to extraction volume |
|---|---|
| Bar | Five pieces of 0.5 × 1 × 0.4 cm/10 ml (0.7 $cm^2$/ml) |
| Film | Twenty-five pieces of 1 × 1 cm/10 ml (5 $cm^2$/ml) |
| Granules | 2 g/20 ml (0.1 g/ml) |

pouring it out from the flask. A blank cell culture medium without sample underwent the same extraction process. The cell culture test was performed with fresh extracts (day 0) and with extracts after 1 or 2 days of storage at 4°C (day 1, day 2).

*Cell Culture Test with Medium Extracts*

A suspension of 10,000 fibroblast cells in 50 μl of growth medium was inoculated in each well of a 96-well microtiter plate. After 1 h of incubation to allow cell attachment to the plate, the medium was carefully removed and quickly substituted with 100 μl of undiluted medium extract. Cells were cultured in the medium extract for 48 h in the incubator. Subsequently, they were subjected to evaluation of cell morphology and viability. Cells cultured in fresh medium (positive control) were used to evaluate the effects of the culturing conditions on cells. Blind values were obtained from medium containing no cells (negative control). Cells cultured in blank, treated medium (Med) served as reference for any change of the medium due to the extraction process.

### Evaluation of Cell Morphology and Viability

*Protein content by the Bradford Method*

The medium in each well was carefully removed. Attached cells were washed with phosphate-buffered saline (PBS). Subsequently, cellular proteins were dissolved by incubation in 0.5 *M* NaOH solution at room temperature for 10 min. Twenty microliters of Bradford dye solution (Bio-Rad protein assay dye reagent concentrate, Bio-Rad Laboratories, 500-0006, containing Coomassie brilliant blue G-250 dye, methanol, and phosphoric acid) was added to each well. On binding to proteins in acidic solutions, the absorption maximum of Coomassie brilliant blue solution was shifted from 465 to 595 nm. After 1 h of reaction, the optical density at 595 nm was measured in a Rainbow-Reader.

*Neutral Red (NR) Uptake*

Twenty microliters of neutral red solution (0.5 mg neutral red in PBS) was added to the cell culture medium in each well. Neutral red was accumulated in the lysosomes of living cells upon incubation for 2.5 h at 37°C. Subsequently, 20 μl Karnovski solution containing 4% formaldehyde and 5% glutaraldehyde was added. The fixation in Karnovski solution lasted for 5 min at room temperature, followed by washing with 100 μl PBS. Neutral red, accumulated in lysosomes, was dissolved for 45 min at room temperature in 100 μl of isopropanol solution containing 0.04 *M* HCl and determined by measuring the optical density at 540 nm. The cellular neutral red uptake was proportional to the lysosomal activity.

*MTT Dehydrogenase Activity*

Ten microliters of MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, 1 mg/ml in phosphate-buffered saline solution; PBS] was added to the cell culture medium in each well. In proportion to the dehydrogenase activity of the cells, water-insoluble formazan was formed from MTT. The plates were incubated for 2 h at 37°C. The MTT solution was then removed and replaced with 100 μl of an ethanol/HEPES solution in each well. The plate was gently shaken and returned to the incubator for another 1–2 h to ensure dissolution of blue formazan. The optical density at 560 nm or the concentration of formazan formed in each well is proportional to the fraction of living cells.

*Phase-Contrast Light Microscopy*

Cell morphology was studied after incubation in extracts and after staining by neutral red or MTT at a magnification of 100 or 200.

## RESULTS AND DISCUSSION

### Evaluation of Cells After Incubation in Material Extracts

Negative effects of the material extracts on cells are indicated by changes in the cell morphology as well as by a decrease in cell growth and viability. The morphology of cells cultured in the extracts can easily be assessed by phase-contrast light microscopy. Cell growth and viability can be performed by various biochemical assays. Total protein content (Bradford method), cellular neutral red uptake, and MTT dehydrogenase activity were determined in this work. Generally, the more vital and active the cells are, the stronger they proliferate. As a result, more cells and a higher total protein content can be achieved. Similarly, the more active, living cells are present, the more neutral red is taken up into cell lysosomes and the more MTT can be transformed in the mitochondria into formazan. The optical density of the solution caused by protein, neutral red, or formazan is theoretically proportional to the growth rate and viability of cells, i.e., proportional to the probability that materials are nontoxic.

*Qualitative Evaluation*

Figure 1 shows fibroblasts in polymer granule extracts after 48 h of incubation (left), followed by neutral red (middle) or MTT staining (right). Since attachment and spreading on the surface of the microtiter plate are necessary for the proliferation of fibroblasts, the toxicity of test material extracts can be estimated by the cell morphology and density. In the cytotoxic PVC extract, fibroblasts maintained a globular form, confirming that cells did not attach, spread, or grow. In the noncytotoxic PE extract, fibroblasts spread well. The typically spindle-shape fibroblasts formed a confluent monolayer on the culture vessel surface.

A neutral red staining (Fig. 1, middle), the contrast was apparently improved. Red spots could be observed inside well-spread cells. These spots are lysosomes where neutral red was taken up by an active transport process through the membrane of living cells. While viable cells can take up neutral red, nonviable or damaged cells are unable to retain the stain intracellularly [5]. In the MTT method (Fig. 1, right), mitochondrial enzyme succinate dehydrogenase within viable cells cleaves the tetrazolium salt (MTT) into a blue-colored product formazan. Blue formazan spots were visible inside well-spread fibroblasts. In addition, needle-like blue crystals of formazan, produced and excreted by living cells into the medium, could be observed repeatedly around spindle-like fibroblasts. In some instances, the contour of single cells cannot easily be discerned in the micrographs due to blue formazan crystals around cells.

Only living cells can take up neutral red or produce blue formazan from MTT, making these methods important vital staining techniques in the evaluation of cell viability. In the medium extract of the plasticized cellulose acetate and PHBV granules, only globular, inactive cells could be observed. Neutral red and MTT staining of these globular, inactive cells could be observed. Neutral red and MTT staining of these globular inactive cells induced, as expected, changes neither in the cell morphology nor in the medium. The medium extract of the plasticized cellulose acetate employed was strongly acidic as indicated by the yellow color of phenol red present in the cell culture medium. The plasticizer used in this cellulose acetate sample was easily extracted and hydrolyzed, producing an acid responsible for the low pH value and cell death [6]. Cellulose powder without plasticizer and possible cytotoxic components in the PHBV extract will have to be identified in a future work.

In the medium extract of Bionolle, besides some globular fibroblasts, most cells were well spread and well stained by neutral red and MTT. In the PCL medium extract, only well-spread fibroblasts were observed. These images demonstrate that phase-contrast microscopy can clearly visualize the morphology of cells cultured in the material extracts, providing valuable indication of possible cytotoxic effects of the test materials.

*Quantitative Evaluation*

*Protein Content by the Bradford Method.* The total protein content was assumed to be proportional to the total cellular material as related to cell growth. Using this method, no distinction can be made between proteins derived from dead and those from living cells. In addition, significantly positive data were measured for the negative controls (neg C) which contain no cells. This deviation from the expected close-to-zero value suggests that the protein determination responds to all, and not specifically to cell, proteins. It is known that cell culture medium contains various proteins, e.g., serum, which may be adsorbed on the cell culture plastic surface. We suspect that the adsorbed proteins can be dissolved in sodium hydroxide solution and react with Bradford reagent, resulting in the positive optical density at 595 nm.

Cell culture medium is formulated for optimal cell growth and proliferation. Cells cultured in the blank medium are expected to yield the maximum of cellular proteins under the given culturing conditions. The greatest difference in the protein content between the blank medium with cells (Med) and without cells (neg C) caused by only a slight change in the optical density. The nonspecific response and slight changes in optical density make protein determination inappropriate for a sensitive determination of cell growth as a function of the test materials.

*Neutral Red Uptake.* The average of the absolute optical density and the standard deviation of a triplicate measurement are presented in Fig. 2. Neutral red, taken up into lysosomes of living cells, was subsequently dissolved and measured at 540 nm. Fibroblasts were cultured in the fresh medium extract of bars (day 0) and in the extract after 1 or 2 days of storage at 4°C (day 1 or day 2).

Neutral red uptake into cells cultured in the extract of the same material shows a random fluctuation independent of the extract storage time. However, in the stored extract (day 2) of PVC, the cytotoxic reference material, neutral red uptake values deviated strongly

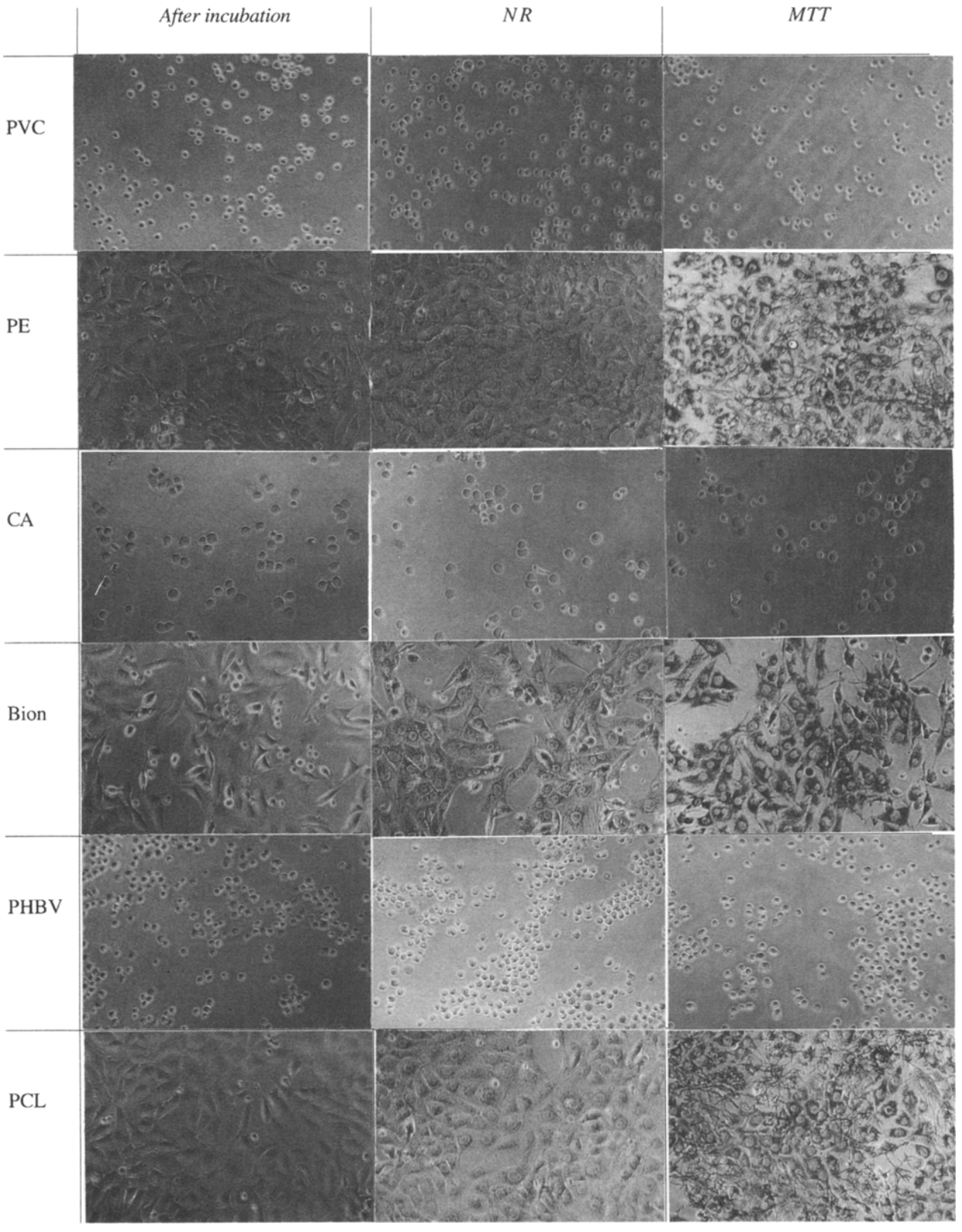


**Fig. 1.** Phase-contrast micrographs (100×) of fibroblasts after 48 h of incubation in extracts (left), followed by neutral red (middle) or MTT (right) staining.

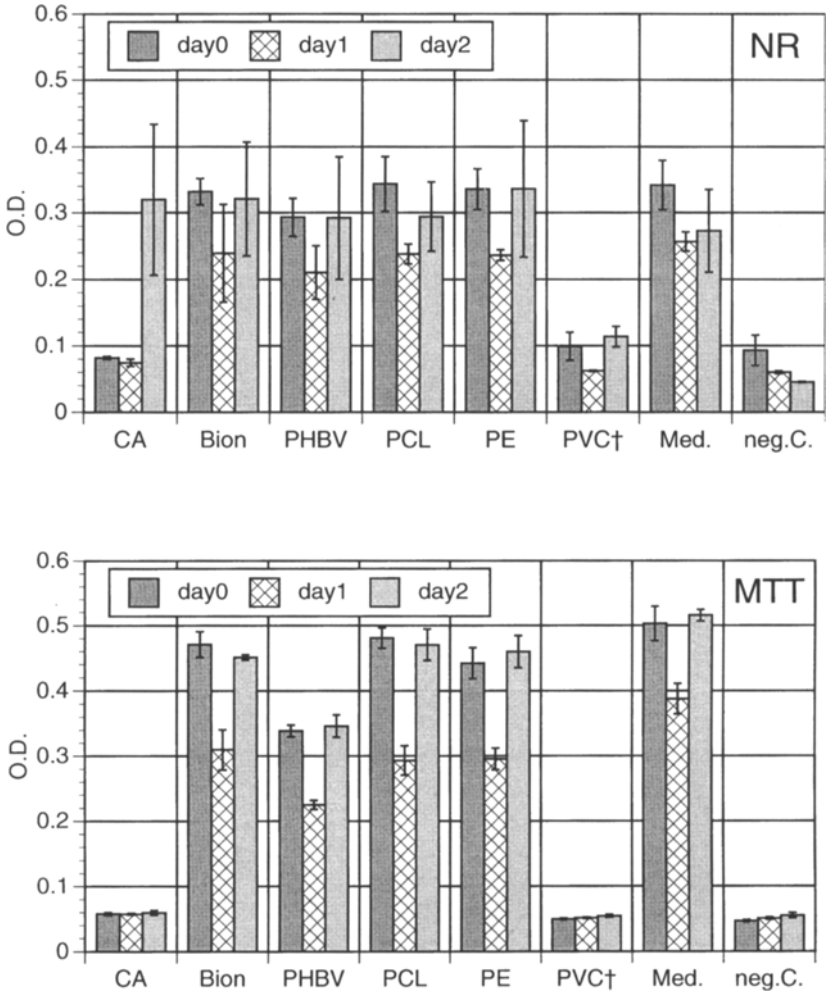


**Fig. 2.** Determination of neutral red uptake and of MTT dehydrogenase activity after incubation of fibroblasts for 48 h in the extracts of bars in the same test series. Medium extracts of bars were prepared fresh (day 0) or after storage at 4°C (day 1, day 2). Three wells were used for each value. Controls were blank, treated medium with cells (Med.) and without cells (neg.C).

from zero, the expected value. The same deviation for extract after 2-day storage was also observed in the CA extract, where cell death was probably due to the acidic medium extract. These deviations suggest that some changes might occur in the medium extract after storage, which can alter the neutral red uptake results.

*MTT Dehydrogenase Activity.* Figure 2 shows that the MTT and neutral red method generally reveal similar effects of the test material extracts on cells. The value obtained from the test with PVC was close to zero as expected, independent of the extract storage time. In addition, the MTT method usually generates an optical density approximately two times higher and a deviation lower compared to the NR method. These observations indicate that the MTT method is more sensitive and reliable than the NR method. It has been mentioned in the literature that the MTT method is a rapid and sensitive technique for the assessment of cytoxicity of materials [7]. Therefore, it is the method of choice for quantitative evaluation of different test materials on cells. To exclude changes during storage, freshly prepared medium extracts (day 0) were used for the evaluation of materials.

## Effects of Materials with Different Shapes on Cells in Tests with Extracts

Sensitive compounds, such as proteins and growth hormones, in the cell culture medium can deteriorate

during 72 h of extraction at 37°C. Blank medium treated under the same conditions can reveal the influence of the extraction process on the medium itself. Therefore, blank treated medium (Med) was used as the 100% noncytotoxic standard for the evaluation of the testing materials to exclude the effects of the extraction process on medium. Based on the relative MTT dehydrogenase activity of cells grown in fresh extracts, materials of the same shape were compared to each other with regard to their effects on cells (Fig. 3). Plasticized CA was not included in this comparison, as the problem with acidic extracts had not been solved.

The difference among test bars, films, and granules seems to be a concentration effect of the substances, which could leach into the extracts. While PCL is comparable to PE in all three forms, Bionolle and PHBV films and granules showed stronger effects on cells than

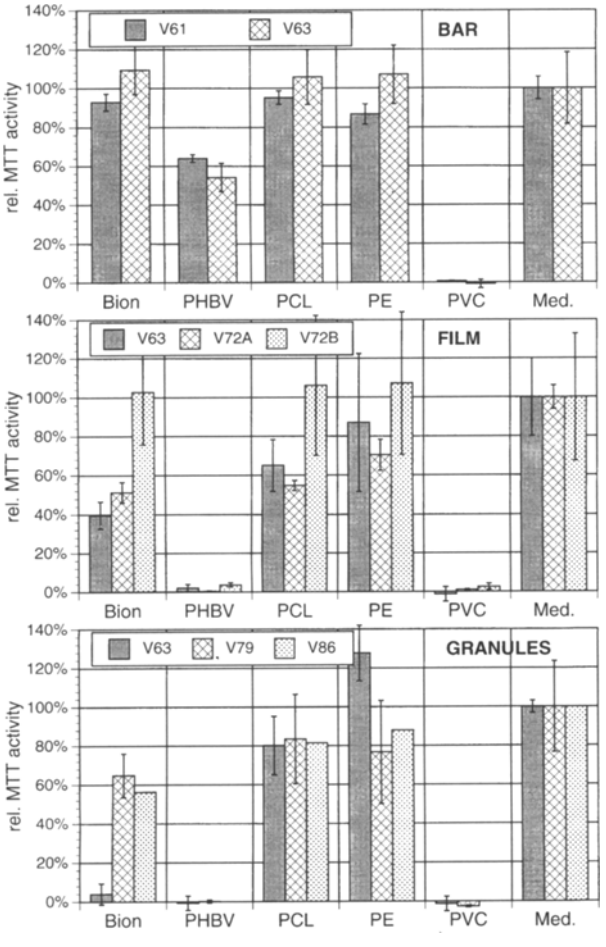


**Fig. 3.** Relative MTT dehydrogenase activity (% of blank, treated medium) of fibroblasts cultured in fresh extracts (day 0) of bars, films, and granules in two or three test series (V). Three wells were used for each value in all series.

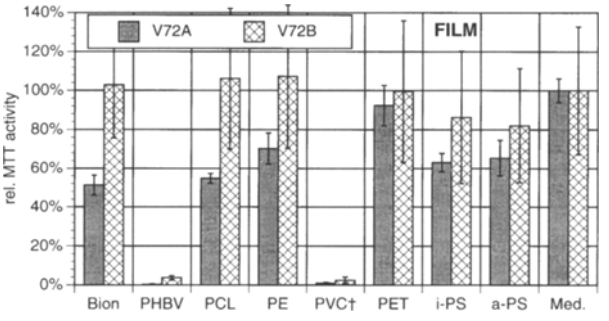


**Fig. 4.** Relative MTT dehydrogenase activity (% of blank, treated medium) of fibroblasts cultured in fresh extracts (day 0) of food-grade plastics and degradable plastics in two test series, V72A and V72B.

corresponding test bars. The difference between bars and films apparently depends on the surface-to-volume ratio of the extraction. The greater this ratio (see Table II), the greater the amount of substances which could be extracted. The difference between films and granules, however, may depend also on the sample weight and composition. Films have a larger surface but a lower weight. In addition, plasticizers and additives present in the granules might evaporate during the film blowing process due to the high temperature. These facts may explain the stronger negative effect of the extracts from granules compared to the extracts from films.

As the amount of leached substances depends on both the surface area and the weight of the test samples in the extraction step, it is necessary to compare materials having the same shape so that these critical ratios can be assumed to be comparable. A material such as PCL shows no cytotoxic effect in all available forms and can be considered noncytotoxic. Other materials such as PHBV can be less cytotoxic in one form, e.g., test bars, but significantly cytotoxic in another form, e.g., films and granules. Therefore, care must be taken to choose the harmless form with respect to a specific application.

Food-grade plasticware—a PET bottle, an i-PS plate, and an a-PS cup—was taken as standard for daily-used materials and investigated simultaneously with the test materials (Fig. 4). The relative MTT dehydrogenase activities of cells cultured in the extracts of these standards and of PCL and Bionolle film were comparable. This result suggests that PCL and Bionolle film may be used as food packaging materials.

## CONCLUSION

The effects of material extracts on fibroblasts can easily be evaluated qualitatively and quantitatively.

Well-spread, spindle-like fibroblasts could be observed in the extract of Bionolle granules. They even formed a confluent monolayer in the extract of PE and PCL. In the extract of cytotoxic reference materials—organic tin-stabilized PVC—only globular cells could be detected. Neutral red staining is preferred to visualize active cells. Of three methods to quantify cell growth and viability—protein assay, neutral red uptake, and MTT dehydrogenase activity—the MTT method showed the highest sensitivity and accuracy. Comparable effects of PCL in all shapes, Bionolle test bars and film, as well as of a PET bottle, PS cup, and plate, on cells in the test with medium extracts indicate that this test can be employed to screen for the safety of new materials in the food industry.

Leachable substances play the main role in the effects of material extracts on cells. These substances are, in most cases, low molecular weight components, such as polymerization initators, cross-linking agent, plasticizers, nucleating agents, processing additives, and degradation products. The cell culture test with extract can be incorporated into the development process of a new plastic material, for instance, in the selection of non-toxic plasticizers and additives.

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