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# Cytotoxicity of some catalysts commonly used in the synthesis of copolymers for biomedical use

M. C. TANZI, P. VERDERIO
*Dipartimento di Bioingegneria, Politecnico di Milano, Italy*
M. G. LAMPUGNANI, M. RESNATI, E. DEJANA
*Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy*
E. STURANI
*Dipartimento di Fisiologia e Biochimica Generali, Università* degli Studi di Milano, Italy

The cytotoxicity of four catalysts commonly used for the synthesis of copolymers for biomedical use, such as segmented polyurethanes, was evaluated towards two types of cells, the first being the well-characterized cell line Swiss 3T3 mouse fibroblasts, the second the actual living system that faces any device in contact with blood, i.e. human endothelial cells (HEC). The catalysts were two tertiary aliphatic amines: TMBDA (tetramethylbutanediamine), and DABCO (1–4 diazo (2,2,2) octane); two alkyl tin compounds: DBTDL (dibutyl-tin-dilaurate), and SnOct (stannous octoate). Cytotoxicity tests were carried out by adding to the culture medium, after cell adhesion, different concentrations of each catalyst in dimethylsulphoxide, and keeping them in contact with the monolayer for 72 h. All the catalysts proved to be cytotoxic, although at different extent (in the order: DABCO < TMBDA < SnOct < DBTDL); their dose inhibiting 50% of cell growth (IC50) came out to be lower for 3T3 fibroblasts than for HEC, with the exception of DBTDL, which showed a similar toxicity for both the cell lines. As an example, the cytotoxicity of a polyurethane-amide, laboratory synthesized with DBTDL as catalysts, was checked with fibroblasts. By using both the method of the extract, and that of the direct contact (through a microporous membrane), a moderate to severe cell growth inhibition, related to the Sn content in the material, was observed.

## 1. Introduction

Tertiary aliphatic amines, and alkyl tin compounds are among the most commonly used catalysts in making linear copolymers, urethane foams, silicones, and in curing reactions of several crosslinked resins.

In the case of segmented polyurethanes, alkyl tin catalysts are specific for the reaction between hydroxyl and isocyanate groups, whereas tertiary amines also catalyse reactions between water and isocyanate groups, leading to urethane foams [1, 2]. Both classes of compounds are endowed with toxicity, and therefore should be eliminated from polymeric materials before their processing, in particular when a prolonged contact with living systems is foreseen. Although the screening of acute and long-term toxicity for both materials and their extractables is recommended by standard practices and protocols, the problem of residual substances used in small quantities for the synthetic step, such as catalysts, is not explicitly taken into account.

Tertiary amines are described as moderate to severe eye and skin irritants, sensitizers and allergens [3]. As for tin compounds, a variable toxicity is pointed out: inorganic derivatives have proved to be essentially harmless, but among the organic ones are strong poisons, and some alkyl tin compounds have high ecotoxicity [3, 4]; moreover, some tin compounds with similar chemical structure have very different toxic effects, and the same compound may be highly toxic to one species and effectively harmless to another [4]. Toxicity data in the literature regard the most commonly used animal species, such as rabbit, mouse and guinea pig, and make reference to the usual administration routes (oral, intravenous, and so on); data on human or specific organic tissues are very poor.

In this work, it was thought of particular interest to evaluate the cytotoxicity of four catalysts, chosen among the most commonly used tertiary amino and tin compounds. These catalysts are listed in Table I, together with their toxicity properties, extracted from the literature.

For the cells, it was decided to test both a well-characterized cell line, i.e. Swiss 3T3 mouse fibroblasts, and freshly isolated human endothelial cells (HEC), which are most likely to become in contact with foreign materials.

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TABLE I Properties of the four catalysts

| Name | Chemical name | Formula | MW | Toxicity data[a] (LD50: mg/kg) |
|---|---|---|---|---|
| DABCO | 1,4 diazabicyclo[2,2,2]–octane | $C_2H_4NC_2H_4NCH_2CH_2$ | 112.18 | LD50(oral, rat) = 1700<br>LD50(oral, rbt) = 1100<br>LD50(oral, gpg) = 2250<br>skin sensitizer |
| TMBDA | N,N,N′,N′–tetramethyl–butanediamine | $(CH_3)_2N(CH_2)_4N(CH_3)_2$ | 144.26 | LD50(oral, rat) = 750<br>LD50(ivn, mouse) = 180<br>LD50(skin, rbt) = 320<br>skin and eye very irritant<br>poison intravenously |
| SnOct | stannous–2–ethylhexanoate,<br>(2-ehtylhexanoic acid, tin(II)salt) | $[CH_3(CH_2)_3CH(C_2H_5)COO]_2Sn$ | 405.1 | [LD50(oral, rat) = 90.7<br>poison by ingestion<br>severe skin irritant][b] |
| DBTDL | dibutyl–bis(lauroyloxy)–stannate | $[CH_3(CH_2)_{10}CO_2]_2$ Sn $[(CH_2)_3CH_3]$ | 631.56 | LD50(oral, rat) = 175<br>LDLo(oral, mouse) = 710<br>LDLo(ipr, rat) = 85<br>poison by ingestion, and<br>intraperitoneal routes. |

[a] From (3): LD50 = lethal dose 50% kill; LDLo = lowest published lethal dose; rbt = rabbit; gpg = guinea pig; ivn = intravenous; ipr = intraperitoneal.
[b] Data on SnOct were not found; these data refer to Di–n–butylin–dibutirrate ($C_{16}H_{32}O_4Sn$, MW = 407.17).

As a practical example, a poly-urethane-amide (PEUAm) [5], synthesized with 2% DBTDL as catalyst at the Hebrew University of Jerusalem was tested for cytotoxicity towards mouse fibroblasts as received, and at different steps of further purification. We used two different experimental procedures: in the former, the cells were grown in the presence of the copolymer, separated from it by a microporous membrane; in the latter, aqueous extracts of the material were directly added to the cultures.

## 2. Experimental procedures

### 2.1. Materials

Tetrametyl-butanediamine (TMBDA, Fluka), 1–4 diazo (2,2,2) octane (DABCO, Fluka), dibutyl–tin–dilaurate (DBTDL, Aldrich), and stannous octoate (SnOct, Sigma) were used as received; before adding them to the culture medium of each cell type, TMBDA and DABCO were dissolved in water, whereas the DBTDL and SnOct were dissolved in dimethylsulphoxide (DMSO).

The poly-urethane-amide (PEUAm) was synthesized as previously described [6] from polytetramethyleneoxide, hexamethylene diisocyanate and fumaric acid, using 2% DBTDL as catalyst, and kindly provided by Dr Cohn (Casali Institute of Applied Chemistry, the Hebrew University of Jerusalem). The PEUAm was isolated as gummy white flakes from the polymerization solution by precipitation in distilled water, without further purification. It was tested as received, and after different steps of purification, by extraction with methanol (24 h under refluxing), or acetone (first extraction 24 h, second extraction 7 days, both under refluxing). The content of Sn in each sample was measured by atomic adsorption analysis (Redox Snc, Cologno Monzese, Italy).

### 2.2. Cell culture

Mouse Swiss 3T3 fibroblasts were grown as previously described [7], in liquid D-MEM medium (Dulbecco-modified Eagle's medium), with 10% fetal bovine serum, 4 mM glutamine, 100 U $ml^{-1}$ penicillin, and 100 $\mu g\,ml^{-1}$ streptomycin, at 37 °C in a 5% $CO_2$ atmosphere.

HEC (human endothelial cells) were isolated from the umbilical vein by treatment with 0.1% collagenase. The culture medium for HEC was medium 199 with 20% newborn calf serum, 50 $\mu g\,ml^{-1}$ endothelial cell growth supplement (ECGS, prepared from bovine brain), 50 $\mu g\,ml^{-1}$ heparin (from porcine intestinal mucosa, Sigma), 50 U $ml^{-1}$ penicillin, 50 $\mu g\,ml^{-1}$ streptomycin, 2.5 $\mu g\,ml^{-1}$ fungizone at 37 °C. The medium was changed twice a week and HEC were passaged (split ratio 1:3) once a week. HEC were used within the fifth passage. All the HEC culture reagents were from Gibco (Grand Island, NY).

### 2.3. Cytotoxicity tests with the catalysts

*3T3 cells.* 1.5 ml portions of a suspension of 3T3 fibroblasts were inoculated in petri dishes of 8 $cm^2$ at a density of 10 000 cells $cm^{-2}$. After 4 h to allow for cell adhesion, 1.5 ml of medium containing the appropriate concentration of the catalyst to be tested was added. Control cultures received the medium additioned of the same volume of DMSO used for solubilize DBTDL and SnOct. After 72 h growth the cells were rinsed with phosphate saline buffer (PBS), exposed to tripsin (0.05% in PBS), and suspended in Isoton (Coulter Electronics, Harpenden, UK). The number of the cells was estimated by a Coulter Counter ZBI. The addition of DMSO to the control cultures did not affect their growth.

*HEC.* Suspensions of HEC were seeded in 96-well tissue cultures plates ($4 \times 10^3$ cells per well). After 4 h

adhesion, the medium was replaced with different concentrations of each catalysts, dissolved, or suspended, in the same medium. Incubation was allowed for 72 h. At this time, the number of cells in the controls was $2.2 \pm 0.2 \times 10^4$.

To evaluate the cellular growth, the culture medium was aspirated, the adherent cells washed twice with $Ca^{++}$- and $Mg^{++}$-free phosphate buffer saline (PBS), fixed with Diff-Quick (Merz Dade, Switzerland) for 3 min, stained with 0.5% Crystal Violet Multiscan (Sigma) for 5 min, and washed twice with distilled water. The plates were scanned by Titertek Multisen (ICN), measuring the adsorbance at 560 nm.

Growth inhibition was calculated in all cases with respect to control cultures. The IC50 (doses inhibiting 50% of cell growth) were determined graphically in a semilogarithmic plot.

## 2.4. Cytotoxicity tests with PEUAm

*Method A.* A cell suspension (1.5 ml) was inoculated in each well of a Millicell culture plate (Millipore) as in section 2.3, at a density of 10 000 cell $cm^{-2}$. After 4 h, Millicell CM membrane units (0.4 μm pores, Millipore) were placed in the wells, and 20 mg of non-purified PEUAm flakes, or 20 mg of Silastic (Lepetit) as reference material, were added, together with 1.5 ml of medium. Control cultures were obtained as in Section 2.3. After 72 h, the cells were counted as described in Section 2.3, by Coulter Counter.

*Method B.* PEUAm flakes (not extracted, MeOH extracted, acetone I extracted, and acetone II extracted) were incubated in 10 ml of culture medium for 72 h at 37 °C, under sterile conditions (portions of 20 mg each). Then, increasing volumes of each extract were tested for cytotoxicity, by diluting them with the appropriate amount of fresh medium (up to 1.5 ml), and adding them to the cell cultures, obtained as described above.

All the tests were done in triplicate, and the average values with standard deviations were then calculated. IC50 values for PEUAm were calculated graphically on a linear plot, with the data obtained from method B, assuming a correspondence between each volume of the extraction media and the quantity of the material.

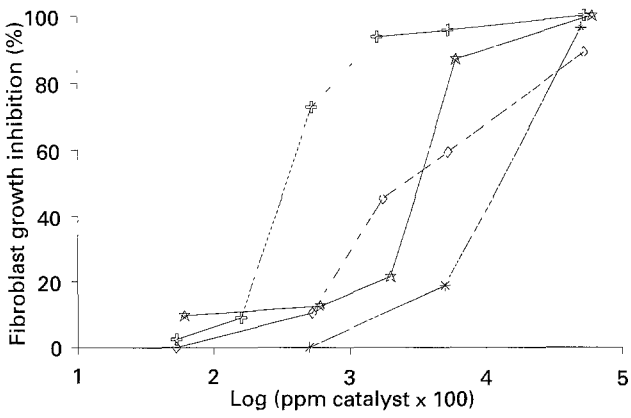


*Figure 1* Growth inhibition of Swiss 3T3 mouse fibroblasts by four catalysts commonly used in copolymerization reactions: ☆ TMBDA tetrametyl-butanediamine; ✳DABCO 1-4 diazo (2,2,2) octane; + DBTDL dibutyl-tin-dilaurate, and ◇ SnOct: stannous octoate (controls = $77.4 \pm 1.2 \times 10^3$ cells $cm^{-2}$, after 72 h growth).

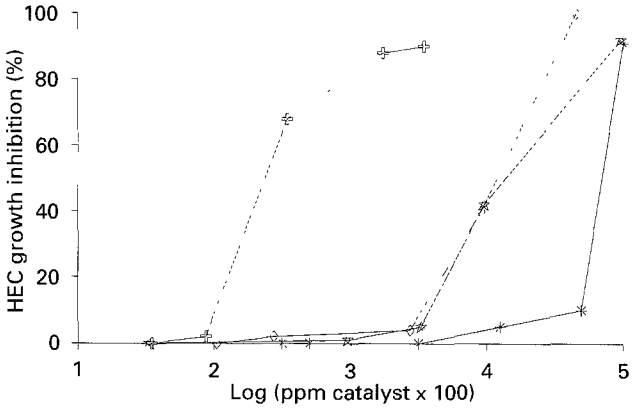


*Figure 2* Growth inhibition of human endothelial cells by the same catalysts as in Fig. 1: + DBTDL; ✳DABCO; ☆ TMBDA; ◇ SnOct.

TABLE II Effect of the four catalysts on cell growth

| Cell type | Catalyst, IC50 (ppm) | | | |
|---|---|---|---|---|
| | DBTDL | SnOct | TMBDA | DABCO |
| HEC | 2.34 | 125.9 | 141.2 | 707.9 |
| Swiss 3T3 | 3.48 | 26.1 | 33.7 | 129 |

## 3. Results

The inhibition of Swiss 3T3 mouse fibroblasts growth, exerted by various concentrations of the four catalysts is shown in Fig. 1, and that of human endothelial cells (HEC) in Fig. 2. Table II reports the corresponding IC50 values.

From these data, it is clear that all the catalysts are cytotoxic, although to different extents. Their cytotoxicity increases in the order: DABCO < TMBDA < SnOct < DBTDL, for both types of cells; this finding is in agreement with *in vivo* data, reported in Table I. In particular, the IC50 of DABCO, TMBDA, and SnOct are lower for 3T3 fibroblasts than for HEC, indicating a higher sensitivity of the former cells. The opposite happens for DBTDL, which shows a higher toxicity for HEC; however, this compound is so toxic, as shown by its IC50 values, that a comparison between the different cell lines is difficult.

The toxicity of DBTDL is also responsible for the results obtained with the copolymer synthesized with this catalyst. As shown in Table III and Fig. 3, the inhibition of fibroblasts growth is dose dependent, and proportional to the Sn content of the sample; therefore, it can be attributed to the presence of residual quantities of the catalyst.

It is also clear that the purification of the copolymer is not easy: the extraction with methanol, a poor solvent for DBTDL, was ineffective in decreasing both the Sn content, and the inhibitory effect, whereas better results have been obtained with acetone, a good solvent for the catalyst; however, even prolonged extraction with a good solvent is unable to completely

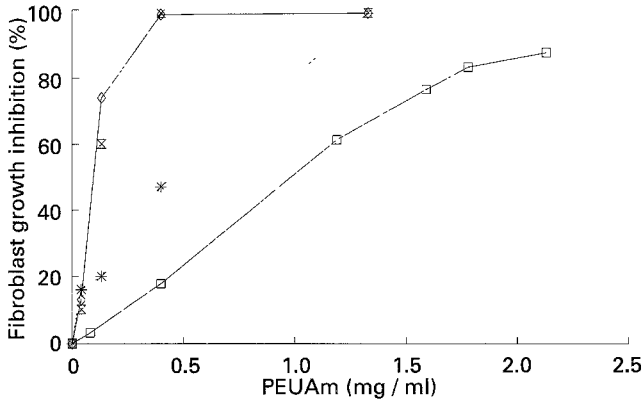


*Figure 3* Growth inhibition of Swiss 3T3 mouse fibroblasts by extracts in culture medium of a poly-urethane-amide (PEUAm) at different steps of purification from residual DBTDL: × not extracted; ◇ MeOH extracted; ✳ acetone extracted (I); □ acetone extracted (II) (controls = $52.5 \pm 2.1 \times 10^3$ cells $cm^{-2}$, after 72 h growth). The values in abscissa are expressed as weight equivalent of PEUAm in the extracting medium.

TABLE III Residual DBTDL content, and IC50 values of differently purified PEUAm samples

| PEUAm samples | Sn (ppm) | DBTDL (% by weight)[a] | IC50 (mg) |
|---|---|---|---|
| Not extracted[b] | 2000 | 1.06 | 0.346 |
| MeOH extracted | 2000 | 1.06 | 0.269 |
| Acetone I extr. | 640 | 0.34 | 1.346 |
| Acetone II extr. | 240 | 0.13 | 2.93 |

[a] Desumed from the respective Sn quantity.
[b] From the experiment under "method A", a dose of 20 mg of this sample had an inhibitory effect of 99%, whereas with the reference material (Silastic) the cell growth was similar to that of the control cultures (controls = $51.5 \pm 6.1 \times 10^3$ cells $cm^{-2}$; Silastic = $57.8 \pm 3.5 \times 10^3$ cells $cm^{-2}$).

eliminate the presence of Sn, which gives a high cytotoxicity even to small quantities of the material. In contrast, it is also evident that the toxic component can be easily released from the material during the contact with the growth medium at 37 °C. Research is in progress to better understand this point.

## 4. Conclusions

From the results obtained, a first conclusion is that the purification of copolymers for biomedical use must proceed very carefully immediately after the synthesis step, particularly when tin compounds are used as catalysts. This is supported not only by the high toxicity shown by the catalysts considered in this work, but also by the difficulty of efficiently extracting them from the copolymer, once isolated and dried.

Another interesting observation that comes out from this work is the different sensitivity of the cells towards noxious agents. As also observed in a previous paper [8], endothelial cells appear more resistant than fibroblasts. This finding suggests the need to take into account the appropriate cell type when testing biomaterials for specific applications.

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