An *in vitro* method for toxicity evaluation of water-soluble substances

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The purpose of the present investigation was to develop a simple method for the evaluation of reversible and irreversible toxic influences in a cell culture system. A cell monolayer established on the bottom of glass scintillation vials was exposed to a toxic substance (phenol). Changes in the DNA synthesis of the cells were utilized as a criterion of toxic influence, and were measured by recording the incorporation of tritium labelled thymidine using a liquid scintillation technique. The exposure of the cells to phenol caused a marked decrease in the rate of DNA synthesis when the phenol concentration was increased from 0.01 to 0.1 %. The decrease in the DNA synthesis could be reversed by maintaining the cells in growth medium for 4 hours after the cell-phenol contact. The degree of reversibility was dependent on the cell-phenol contact time, the phenol concentration, and the cell line used. The simple test procedures and the quick and convenient obtainment of results simplify the assay of large test series and make the method particularly useful for screening tests.

Key-words: Tissue culture; DNA; radioisotopes; toxicology

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In the development of a toxicity evaluation program for dental materials and products, evaluation of cytotoxicity in mammalian tissue culture systems has appeared suitable as preliminary screening tests (*Powell et al.*, 1970; *Spångberg*, 1973). Different techniques have been applied to evaluate cytotoxic influences on cultured cells (for review of the literature, see *Rofe*, 1971). Evaluation methods which combine simplicity and rapidness have been introduced by *Guess et al.* (1965), who utilized a vital stain exclusion technique, and by *Spångberg* (1973), who measured the release of a radioactive isotope incorporated into the cells. These methods, however, do not allow the evaluation of a possible reversibility of the cytotoxic influences on the cells. It seems likely that if changes in metabolic activity are used to evaluate the cytotoxic influences on the cells, reversible and irreversible damages to the cells may be distinguished (*Milner*, 1967). A commonly used method to evaluate metabolic activity in a tissue culture system is to measure the DNA synthesis of the cells (*Seed*, 1965). Since DNA has practically no turnover, incorporation of a precursor into the DNA molecule may be considered as a sign of synthesis of this molecule (*Firket & Verly*, 1958). Thymidine has been shown to be a specific precursor of DNA (*Friedkin, Tilson & Roberts*, 1956) and

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the uptake of radioactive thymidine into the cell nucleus may be used as a measure of DNA synthesis (*Stanners & Till*, 1960).

The purpose of the present investigation was to study if changes in metabolic activity may be used to distinguish between reversible and irreversible cytotoxic influences on the cells, and if so, whether these changes could be utilized as a suitable measure of toxicity in a simple test system.

MATERIALS AND METHODS

1. Cell lines and culture technique. Mouse fibroblast cells (L929, Flow Laboratories, Irvine, Scotland) were maintained as stationary glass cultures in Eagle's minimum essential medium (Eagle's MEM, *Eagle*, 1959) supplemented with 10 per cent fetal bovine serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin. Human epithelial cells (HeLa, Flow Laboratories) were maintained as stationary glass cultures in Eagle's MEM supplemented with 5 per cent fetal bovine serum, 5 per cent calf serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin. The cultures were subcultivated twice a week. The HeLa cells were harvested with 0.02 per cent (w/v) Versene (Flow Laboratories) in Dulbecco's phosphate buffered saline (PBS) (*Dulbecco & Vogt*, 1954) without calcium or magnesium. The L cells were harvested with 25 μg/ml crystalline trypsin (Trypure®, Novo, Copenhagen, Denmark) in PBS without calcium or magnesium.

After 6 subcultivations a portion of the cells was frozen according to the method described by *Paul* (1973) and stored in a liquid nitrogen refrigerator. At the same time samples of cells and culture medium were taken to be examined for mycoplasma contamination according to the agar culture method of *Hayflick* (1965). Mycoplasma grew in no culture. To minimize the hazard of culture alteration during the experiments, the cells were discarded when they had been maintained as stationary glass cultures for a period of approximately 6 months. New cells were obtained from the frozen stock. Before a cell culture was discarded samples were taken for mycoplasma culturing. Mycoplasma grew in no culture.

2. Inoculation of cells. Two days after subculture, cells from the stock cultures were harvested and suspended in culture medium. Cell counts were made in a haemocytometer chamber under phase contrast microscopy (*Paul*, 1973), and the suspension was diluted in culture medium to provide the wanted cell concentration. The cell suspension was placed on a magnetic stirrer and with the aid of an automatic pipette aliquots of the cell suspension were transferred to glass scintillation vials (Labora, Stockholm, Sweden). 1.8 ml of the cell suspension was dispersed into each vial, which served both as culture vessel and test vessel. To establish a cell monolayer on the bottom of the vials, they were incubated for 18 hours at 37°C in a humidified atmosphere of 5 % CO_2 in air. When the time required for the test itself exceeded 8 hours the incubating time was shortened so that the total experimental time did not exceed 26 hours.

To determine an adequate cell inoculation density of the scintillation vials, test vials were inoculated with cell suspensions of various cell densities. After being cultured for 26 hours the DNA synthesis of the cells was studied.

3. Protein measurements. To appraise the accuracy of the dispersion of the cells, protein measurements were performed. From a cell suspension with known cell

concentration (determined by counting in a haemocytometer chamber) 10 one ml aliquots were transferred to test tubes using the earlier described dispersion technique, and the total protein content of each tube was measured. This procedure was repeated 5 times with various cell suspensions.

From a cell suspension containing 3×10^5 cells per milliliter, 10 scintillation vials were inoculated according to the previously described dispersion technique. After incubation of the vials for 26 hours the total protein content of each vial was measured. This procedure was repeated 3 times. The protein concentration of the cells in the scintillation vials was determined spectrophotometrically (Beckman® DB-G, Beckman Instruments, Geneva, Switzerland) according to the method described by *Oyama & Eagle* (1956). The protein content of the cell aliquots in the test tubes was measured using the same procedure exept that the cells were sedimented by centrifugation (10 min, 800 g) prior to and after each wash.

4. Synthesis of DNA. The DNA synthesis of the cells was evaluated by measuring the radioactivity of the extracted DNA after the cells had been incubated with a radioactive DNA precursor (Thymidine-6-^{3}H, ^{3}H-TdR, specific activity 5 Ci/mmol, Radiochemical Centre, Amersham, England). The DNA was extracted by the method of *Schneider, Hogeboom & Ross* (1950), adapted to cells attached to a surface (*Ball, Poynter & van den Berg*, 1972).

5. Test solution. Stock solutions were prepared by mixing crystalline phenol (Kebo, Stockholm, Sweden) with glass destilled water. All stock solutions were prepared at the same time and kept frozen until used. The desired test concentration was obtained by adding 0.2 ml of a 10 times stronger stock solution to the 1.8 ml of culture medium already in the vial. In the controls PBS was substituted for the phenol solutions.

6. Test procedure. When a monolayer was established on the bottom of the vials, the stock solution of phenol was added to the culture medium, hereafter called the test medium, for time periods of 2 and 12 hours. Two different procedures were then applied. (1) ^{3}H-TdR, at a concentration of 0.5 μCi/ml, was added directly to the test medium 30 min before the end of the time interval chosen for the influence of the test medium on the cells. After a ^{3}H-TdR pulse of 30 min the radioactivity incorporated into the cells was measured. (2) At the end of the time interval chosen for the influence of the test medium, this was withdrawn and the cell monolayer washed twice with prewarmed culture medium without serum. Two ml of prewarmed culture medium was then added to each vial which was further incubated for 4 hours. Upon the incubation, ^{3}H-TdR (0.5 μCi/ml) was added to the culture medium. The incorporated radioactivity was measured after 30 min.

7. Radioactivity measurements. At the end of the incorporation time, the radioactive medium was withdrawn from the vials and the cell monolayer washed 3 times with ice-cold perchloric acid solution (PCA, 1.5 % v/v). After adding 0.7 ml of 5 % PCA to each vial they were kept at 90°C for 30 min. After cooling, 10 ml of a scintillation fluid consisting of analytical grade toluene (BDH Chemicals, London, England), 5 g/l 2.5-diphenyloxazole (PPO, BDH Chemicals), 0.3 g/l 1.4-bis-2-(4-methyl-5-phenoloxazoylyl)-benzene (POPOP, BDH Chemicals) and 10 % (v/v) Beckman® Solubilizer BBS-3 (Beckman Instruments) was added to

each vial and the radioactivity recorded in an LKB-Wallac model 81000 liquid scintillation counter (Wallac, Turku, Finland).

To evaluate the reliability of the radioactive measurements, the following modification was utilized. At the end of the incorporation time, the radioactive medium was withdrawn from the vials and the cell monolayer washed with 4 ml of ice-cold 1.5 % PCA. One ml of the 1.5 % PCA was transferred to a scintillation vial for radioactivity recording, the rest was discarded. This procedure was repeated 3 times. Then 4 ml of 70 % ethanol was added to the vials. After 10 min one ml of the ethanol was taken to radioactivity recording and the rest discarded. Two ml of 5 % PCA was then added to the vials which were kept at 90°C for 30 min. After cooling, the vials were centrifuged for 10 min at 800 g. Of the supernatant 0.5 ml was taken to radioactivity recording and the rest discarded. The cell pellet was resuspended in 2 ml of 5 % PCA and the above procedure repeated. The cell residue was solubilized by resuspending the cell pellet in 2 ml of 0.2 N NaOH after which the suspension was kept at 90°C for 2 hours. After cooling, the vials were thoroughly shaken and 0.5 ml of the sodium hydroxide solution taken to radioactivity recording. The values of the radioactive intensity (CPM) recorded in the different fractions were multiplied by 4 and then compared with the CPM value of the first hot PCA fraction.

Counting efficiency was determined by the external standard ratio method from a standard quenching curve, prepared by using quenched samples of known radioactivity (*Davies & Hall*, 1969). Correction for quenching effects was found necessary only in the study of the reliability of the radioactive measurement procedures. The background radiation never exceeded 0.1 % of the radioactivity of the control samples and was neglected. Counts attributed to adsorption of tritium to the scintillation vials were considered constant and were not subtracted from the recorded counts.

8. Statistical methods. Concentional statistical methods were used for calculation of means, standard deviations and coefficients of variation. The linear relationship between two variables was studied by product-moment correlation. In the phenol experiments the mean recorded radioactive intensity of the experimental group (CPM_e) was expressed as per cent of the mean recorded radioactive intensity of the control group (CPM_c). The error of mean of the CPM_e-values was calculated using Gauss' approximated formula (*Blom*, 1970).

RESULTS

1. *Evaluation of test procedures*

1.1. Reliability of the radioactivity measurement procedures. The results are summarized in Table I. The CPM values represent the mean of three tests and quenching corrections have been made.

Practically all nonincorporated radioactivity was removed from the cells after 2 washes of the cell monolayer with cold 1.5 % PCA. The radioactivity recorded in the first hot PCA fraction was 103149 CPM. In the second hot PCA fraction the radioactivity was only 2.2 % of that measured in the first fraction. The radioactivity of the solubilized cell residue was negligible.

1.2. Cell inoculation density. The relationship between cell inoculation density and ^{3}H-TdR incorporation into the cells 26 hours after plating is shown in Fig. 1.

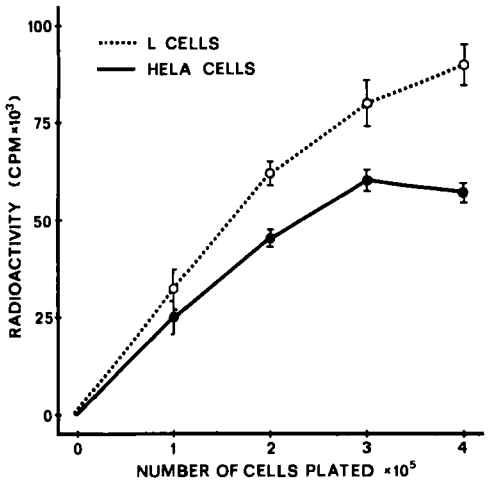

Fig. 1. Incorporation of ^{3}H-thymidine into DNA of L cells and HeLa cells during 30 min pulse after 26 hours culturing in glass scintillation vials. Mean ± S.D. (n=5). A linear relationship appears to exist between incorporated radioactivity and number of cells plated up to a cell inoculation density of 3×10^5 cells per vial.

Table I. *Radioactivity of the different fractions of the nucleic acid solubilization procedures in absolute values (CPM) and in per cent of the radioactivity of the first hot PCA extraction. Mean of 3 tests*

	CPM	%
1st. wash with cold PCA	15,076	14.6
2nd. wash with cold PCA	1,649	1.6
3rd. wash with cold PCA	339	0.3
4th. wash with cold PCA	167	0.2
Alcohol extraction	293	0.3
1st. hot PCA extraction	103,149	100.0
2nd. hot PCA extraction	2,299	2.2
Solubilized cell residue	550	0.5
Background radiation	27	<0.1

Table II. *Protein contents of one ml cell aliquots dispersed from 6 different cell suspensions* (n=*10*)

Cells per ml (x 10^5)	Protein μg $\bar{x}$	S.D.	Coefficient of variation
1.0	31.5	1.4	4.6
1.0	34.9	1.7	4.9
3.0	101.6	4.2	4.1
3.0	95.6	6.6	6.9
6.0	216.2	11.1	5.1
6.0	213.8	7.5	3.5

Table III. *Protein contents of cell aliquots dispersed from 4 different cell suspensions and cultured for 26 hours on the bottom of glass scintillation vials (n = 10)*

Number of cells plated (x 10^5)	Protein μg $\bar{x}$	S.D.	Coefficient of variation
3.0	264.3	9.2	3.5
3.0	217.9	5.6	2.5
3.0	275.3	25.7	9.3
3.0	204.8	7.7	3.8

A linear relationship appeared to exist between incorporated radioactivity and cell number for both HeLa cells (r = 0.945) and L cells (r = 0.959) up to a cell inoculation density of 3×10^5 cells per vial. For practical reasons 1.8×10^5 cells per vial was chosen as cell inoculation density.

1.3. Cell inoculation procedure. Non-cultured cell aliquots dispersed from the same cell suspension showed only minor variations in protein content (Table II). In 6 experiments, each comprising 10 aliquots the coefficient of variation of the protein content ranged from 3.5 % to 6.9 %, averaging 4.8 %. A linear relationship was found between cell count and protein content (r = 0.995).

Cell aliquots dispersed from the same cell suspension and cultured for 26 hours before protein determination showed the same small variations in the protein content (Table III). The coefficient of variation in 4 experiments ranged from 2.5 % to 9.3 %, averaging 4.8 %. No correlation was found between cell count and protein content in this experimental series.

2. Phenol tests

2.1. 2 hours cell-phenol contact. The results are summarized in Fig. 2. A continuing decrease in the ^{3}H-TdR uptake into the

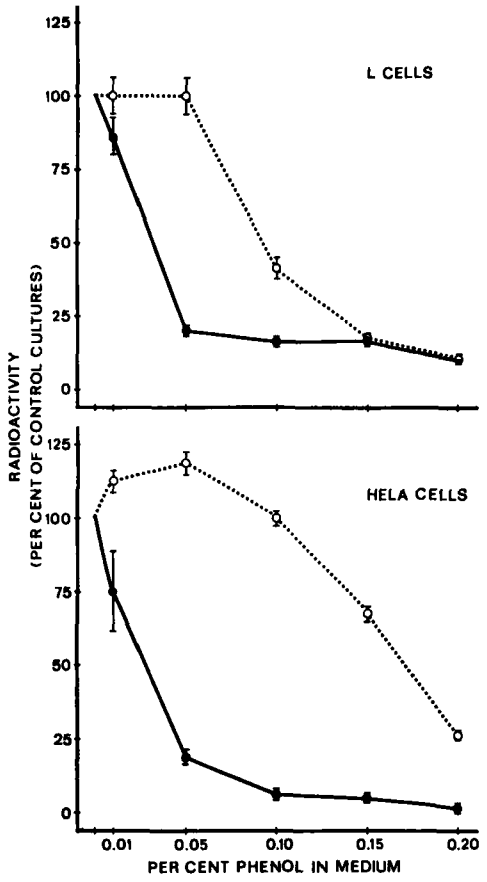

Fig. 2. Incorporation of ^{3}H-thymidine into DNA of L cells and HeLa cells during 30 min pulse after 2 hours cellphenol contact time (solid line), and 2 hours cellphenol contact time followed by 4 hours in growth medium (broken line). The mean values ± S.D. (n = 5) are expressed as a percentage of the incorporation recorded in the control cultures.

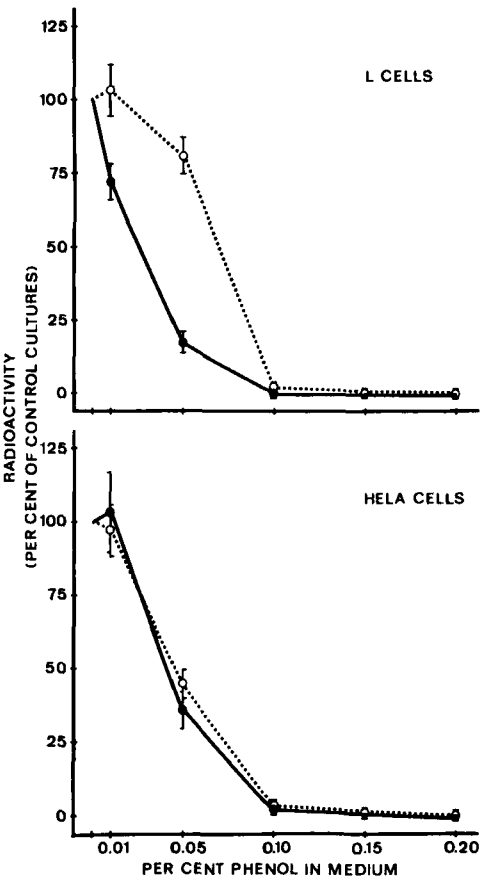

Fig. 3. Incorporation of ^{3}H-thymidine into DNA of L cells and HeLa cells during 30 min pulse after 12 hours cellphenol contact time (solid line), and 12 hours cellphenol contact time followed by 4 hours in growth medium (broken line). The mean values ± S.D. (n = 5) are expressed as a percentage of the incorporation recorded in the control cultures.

cells was observed for both L cells and HeLa cells when influenced by phenol in concentrations up to 0.10 %. At this concentration the ^{3}H-TdR uptake into the L cells was 15 % of the control, and the uptake into the HeLa cells 7 % of the control. An increase in the phenol concentration to 0.20 % caused only minor changes in the ^{3}H-TdR uptake.

When the cells were maintained in growth medium for 4 hours after the phenol influence, the HeLa cells showed an increase in the ^{3}H-TdR uptake, compared to the control, at phenol concentrations of 0.01 % and 0.05 %. An increase in the phenol concentration caused a continuing decrease in the ^{3}H-TdR uptake into the HeLa cells to 27 % of the control at a phenol concentration of 0.20 %. L cells, maintained in growth medium for 4 hours after influence of phenol concentrations of 0.01 % and 0.05 %, showed no change in the ^{3}H-TdR uptake as compared to the control. When the phenol

concentration was increased, a continuing decrease in the ^{3}H-TdR uptake into the L cells was observed. At a phenol concentration of 0.20 % the ^{3}H-TdR uptake was 10 % of the uptake of the control.

2.2. 12 hours cell-phenol contact. The results are summarized in Fig. 3. Both L cells and HeLa cells showed a decrease in the ^{3}H-TdR uptake when influenced by phenol in concentrations up to 0.10 %. At this concentration the ^{3}H-TdR uptake into both L cells and HeLa cells was completely inhibited. The inhibition was not reversed by maintaining the cells in growth medium for 4 hours after the phenol influence. At phenol concentrations of 0.01 % and 0.05 %, keeping of the cells in growth medium for 4 hours after the cell-phenol contact, reduced the decrease in ^{3}H-TdR uptake into the L cells, but did not alter the ^{3}H-TdR uptake pattern of the HeLa cells.

DISCUSSION

The various procedures involved in the present test method were feasible and fairly easily accomplished. For instance, by culturing the cells directly on the bottom of glass scintillation vials it was possible to incorporate the labelled precursor and carry out the recording of the radioactivity in the same vial. This simplified the recording procedures (*Miller, Walker & Giblak*, 1972), but necessitated that all nonincorporated radioactivity was removed from the vials before recording, and that the incorporated radioactivity was limited to the DNA fraction of the cells (*Ball et al.*, 1972). The evaluation of the radioactivity measurement procedures revealed that 3 washes of the cell monolayer with cold 1.5 % PCA removed the nonincorporated radioactivity adequately. The PCA washes also fixed the cells and attached them to the bottom of the vials (*Ball et al.*, 1972). Ethanol extraction was omitted since it removed only traces of radioactivity and because the counting technique used did not require dehydration of the test samples. It appeared that one hot PCA extraction was sufficient, since, in a second hot PCA extraction, the recorded radioactivity constituted only 2.2 per cent of the radioactivity recorded in the first hot PCA extraction. Following 2 hot PCA extractions, the cell residue left in the vials contained only traces of radioactivity, thus confirming that the incorporated radioactivity was limited to the nucleic acids.

The evaluation of the radioactivity measurement procedures thus indicated (1) that the nonincorporated radioactivity was adequately removed, (2) that one hot PCA treatment of the cells sufficiently extracted the incorporated radioactivity, and (3) that the cell residue left in the scintillation vials after the hot PCA extraction contained only negligible radioactivity.

It should be born in mind that this method does not measure DNA specific radioactivity, but the total thymidine incorporation into the cells. However, incorporation of thymidine has been found to be proportional with the number of cells when the incorporation occurred 24 hours after plating, and the cell number did not exceed the density-dependent inhibition concentration (*Ball et al.*, 1972). The results of the evaluation of the cell inoculation density indicated that these conditions were fullfilled in the present study.

Since the thymidine incorporation under the conditions tested was proportional with the number of cells, comparison between the rate of DNA synthesis of the

different test samples may be performed provided that all test samples contained the same number of cells at the beginning of the test, and were cultured under similar conditions.

In a pilot study which was carried out in order to evaluate an applicable method to disperse the cells uniformly into the vials, the total protein content of the dispersed cell aliquots was utilized to establish the variation in cell number of each aliquot (*Oyama & Eagle*, 1956; *Holden, Lichter & Sigel*, 1973). The protein concentration per cell has been found to vary with the number of days in culture, with media conditions, and with the growth phase of the cells (*Salzman*, 1959; *Absher*, 1973). In the present study, therefore, the determination of the protein content was performed on cell aliquots dispersed from one cell suspension at the same occasion, and accordingly, the protein concentration of the cells could be assumed to be the same.

The results of the protein determinations indicated that a satisfactory uniformity in cell dispersion was achieved with the utilized dispersion proceedings. Even after 26 hours incubation the variance in the total protein content of each vial inoculated from the same cell suspension was small, and the culture conditions thus appeared satisfactory. However, vials inoculated from different cell suspensions with the same cell concentration showed a difference in the protein content, indicating a difference in cell number. Since a good correlation was found between cell count and protein count immediately after the dispersion of the cells, the difference in protein content after 26 hours culturing most likely was due to a difference in growth ability of the cells in different suspensions. Therefore, to allow comparison between experimental series where the test samples were inoculated from different cell suspensions, the recorded incorporation of thymidine had to be converted to a relative percentile figure based on the incorporation in the control. This obstructs the possibility to calculate the absolute toxicity of different substances. However, the absolute rate of DNA synthesis cannot be measured by recording of thymidine uptake only (*Smets*, 1969), and consequently the method should in any case be restricted to the calculation of relative toxicity.

The ^{3}H-TdR incorporation during influence of phenol showed approximately the same pattern for HeLa cells and for L cells. An increase in the phenol concentration caused a decrease in the^{3}H-TdR uptake. Variations in the phenol concentration of less than 0.05 % could be demonstrated by differences in the ^{3}H-TdR uptake into the cells.

A possible reversibility of the decrease in the ^{3}H-TdR uptake into the cells was evaluated by recording the ^{3}H-TdR uptake 4 hours after interruption of the cell-phenol contact. It appeared that the decrease in the ^{3}H-TdR uptake could be reversed, and that the degree of reversibility was dependent on (1) the cell-phenol contact time (2) the phenol concentration, and (3) the cell line.

The increase in the ^{3}H-TdR uptake into the cells, compared to the controls, which was noticed in some of the experiments, and the difference in degree of reversibility between HeLa cells and L cells may have various explanations. Complementary studies are necessary to elucidate these findings. Possible explanations may be that the phenol interferes with the synthesis of thymidine monophosphate, that it blocks the cells from entering the DNA synthesizing phase, or that it causes a slow-down in the rate of DNA synthesis.

From the results of the present study it may be concluded, that the described procedures may constitute a practicable method to evaluate cytotoxic influences of water-soluble substances in a cell culture system. Especially it should be noted, that a possible reversibility of the cytotoxic influence may be revealed and that the degree of reversibility may be evaluated. The quick and convenient obtainment of results and the minimal handling of the cells which remain in the same vial during the whole test procedure simplify the assay of large test series and may make the method particularly useful for screening tests.

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