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APPLICATION OF CELL CULTURE AS A PRIMARY TOXICITY SCREEN OF POSSIBLE SPACECRAFT CONTAMINANTS

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SUMMARY

Cell culture has been investigated with regard to its applicability as a primary toxicity screen. Forty-nine compounds have been screened by this method. These compounds are all contaminants which may occur in manned spacecraft. The data presented indicate that cell culture can be a useful tool for selecting, from a long list, those compounds most likely to be toxic to a living system. The compounds tested might be ranked, in terms of decreasing toxicity to cells in culture, as follows: unsaturated aldehydes, amines, aldehydes, acids, ketones, and alcohols.

INTRODUCTION

In any closed life-support system, there will be evolved many substances not normally present at significant concentrations in the earth's atmosphere. In that sense, these substances may be referred to as contaminants. These contaminants evolve as metabolic byproducts of the living system enclosed as well as from the materials which constitute the enclosing system. (See ref. 1.) In a manned spacecraft on an extended mission, these contaminants may rapidly build up to toxic levels (ref. 2) if they are not removed. Because of the obvious weight restrictions involved in any spacecraft design, an absolute contaminant removal system would seem to be impractical. Therefore, some balance must be achieved between the weight penalty for a contaminant-removal system and the concentration of contaminant that may be permitted to remain in the atmosphere of the spacecraft. The achievement of this balance requires an investigation into the relative toxicities of the various contaminants evolved from the personnel and material in the spacecraft.

Presently, most toxicity data are derived from investigations involving whole animals, and these investigations may take from several weeks to several months to yield definitive data. Because of the large number of contaminants identified from manned testing in simulated space cabins (refs. 2 and 3), submarine studies (ref. 1), Mercury and Gemini missions (refs. 1 and 4), and in materials degradation studies, whole-animal evaluation of these products would require an extensive and prolonged investigation.

To accelerate the availability of needed data, therefore, it is advantageous to develop some means for rapidly screening these contaminants in order to obtain a first-order approximation of their toxicities.

The American Conference of Governmental Industrial Hygienists (ref. 5) has listed threshold-limit values for those compounds of industrial interest. These threshold limit values refer to the concentration in air believed to be tolerable to men exposed 8 hours per day, 5 days per week. It should be noted that the techniques used in the establishment of threshold-limit values are by no means constant between compounds. They are established by a number of workers in various laboratories using a variety of methods. In general, there is no common denominator for comparing the relative toxicities of compounds investigated in different laboratories. The criteria for establishing these values are based on physical irritation in some cases and physical impairment in others.

Cell cultures are similar to undifferentiated cells in vivo and should respond in a qualitatively similar manner to most toxic contaminants. Any contaminant that affects most tissues in vivo would be expected to elicit an observable reaction in cell culture. It is for this reason that cell culture is adaptable as a primary toxicity screen.

Cell culture has been used by a number of workers (refs. 6 to 8) as a means of screening pharmaceutical chemicals for their cytotoxic effects. The primary objective of these investigations has been, however, to screen for cancer chemotherapeutic agents in terms of differential toxicity and not for the relative toxicities of these substances.

Rightsel's (ref. 9) development of a technique for studying viruses in cell culture utilizing vinyl plastic containers has been very useful in simplifying the use of cell culture for studies of cytotoxicity. Use of a modification of this technique similar to that of Toplin's (ref. 7) should make preliminary toxicity studies rapid, reproducible, and relatively inexpensive, especially when compared with whole-animal investigations.

An adaptation of this technique was used in an investigation into the relative toxicities to HeLa cells in culture of forty-nine contaminants which may occur in spacecraft. These results are reported in this paper. There has been no attempt to relate absolutely the toxicity in cell culture with the toxicity in man. The primary purpose of these investigations has been to establish the feasibility of the use of cell culture to obtain a first-order approximation of the toxicity for the substances tested.

MATERIALS AND METHODS

The HeLa cells utilized in these experiments are an established cell line originally derived from a human carcinoma of the cervix. Stock cultures of these cells were obtained from the Cell Repository of the American Type Culture Collection in Rockville,

Maryland (HeLa cells: certified cell line 2). These stocks were serially subcultured at least twice in this laboratory before experimentation to eliminate any effects from shipping and freezing and to adapt the cells to the medium used in this laboratory. The cells were grown in antibiotic-free Eagle's minimum essential medium supplemented with 10-percent calf serum. Routine checks were made on the cultures for bacterial contamination by using tryptic soy broth and agar. All cultures were periodically examined for the presence of pleuropneumonia-like organisms (PPLO), a common microbial contaminant of cell cultures, by using the technique of Fogh (ref. 10). Briefly, this technique involved hypotonic treatment of the culture under study, air drying, and staining with orcein. The preparation was then examined directly with the use of bright-field microscopy.

The contaminants were screened by a modification of a technique of Toplin (ref. 7). This procedure involved the use of disposable plastic cups (see fig. 1), each with an approximate volume of 1.5 ml. These cups were sterilized overnight in 70-percent ethanol, dried under an ultraviolet light, and stored in sterile glass tubes. Depending upon the contaminant involved, a fivefold dilution series of a variable number of cups was used in studying the effects of each contaminant. The work being done in a cell-culture handling box, 0.75 ml of a suspension of cells in the medium was added to the first cup in the series and 0.8 ml added to the other cups. The cell concentration in the suspension was approximately 60 000 per ml. Aseptically, 0.25 ml of contaminant was added to the first cup to yield a final concentration of 250 000 parts per million (ppm) on a volume basis in that cup. A serial dilution of 0.2 ml of the thoroughly mixed contents of this first cup was made through the rest of the cups in the series for each contaminant.

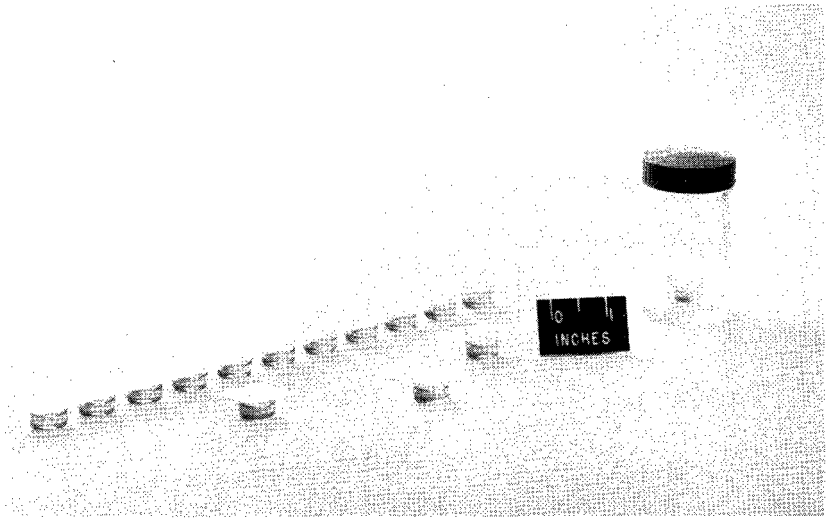

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Figure 1.- Vinyl plastic cups used in the dilution series.

One extra cup was used as a control for any contaminant that might diffuse into the experimental cups from the contaminant container during the handling procedure. Table I shows the contaminant concentration in each cup in a fifteen-cup series. Some of the contaminants were not soluble in the medium at a concentration of 250 000 ppm. In this case, 0.95 ml of cell suspension and 0.05 ml of contaminant were added to the first cup in the series and this cup was then considered equivalent to the second cup in the other series. In the case of methanal (formaldehyde), where the maximum concentration available in solution was 37 percent, 0.14 ml of this solution was added to 0.86 ml of cells in the medium in the first cup in the series. This cup was then considered equivalent to cup 2 in the series involving other contaminants. No attempt was made to control the absolute number of cells contained in each of the cups since the criterion for toxicity evaluation involved only the morphology of the cells and not the quantity. In all cases, duplicate series were run for each contaminant. Subsequent to the dilution of the contaminant through the series, the cups were sealed with pressure-sensitive cellophane tape and each cup placed in a separate container to prevent diffusion of the contaminant between cups. This step was shown to be necessary in preliminary work. The containers were then incubated for 48 hours at 37° C (±0.5° C).

TABLE I.- CONCENTRATION OF CONTAMINANT IN EACH CUP IN DILUTION SERIES

Cup	Contaminant concentration, ppm
1	250 000
2	50 000
3	10 000
4	2 000
5	400
6	80
7	16
8	3.2
9	.6
10	.1
11	.03
12	.005
13	.001
14	.0002
15	0.0 (control)

As a control for the effects of handling the cells, the cell suspension with no additions was mixed with a pipet in a manner identical with the mixing in the contaminant series. This handling was done in several series of cups. The effect of dilution of the medium was studied by using a serial dilution of sterile distilled water in the same manner as the contaminants were used.

After 48 hours, the cups were removed from their containers and the cells in each examined with the use of phase-contrast microscopy. The cups were individually scored (that is, given a toxicity rating) on the basis of the morphology of these cells. The scoring was as follows (see fig. 2):

Toxicity rating	Appearance
0	No change in the appearance of the cells as compared with control groups.
1	Cell density appears less than that in the control groups; cells slightly rounded and some free in the medium but most still attached in the normal manner.
2	Cells mostly suspended and clumped; rounding of cells pronounced; few cells still attached.
3	All cells suspended; cells irregular in shape; much cell debris present; cells incapable of growth in fresh medium.

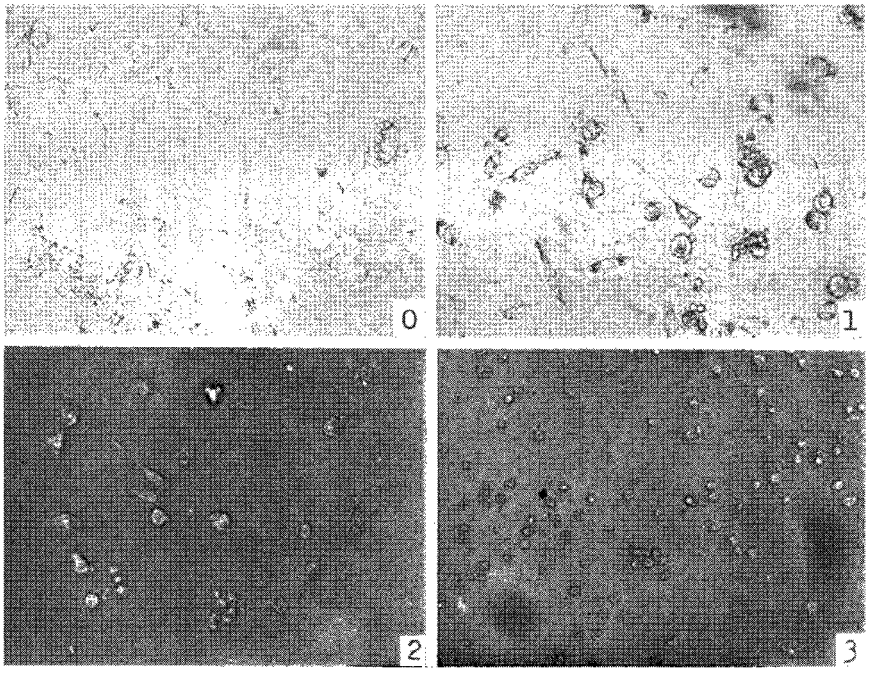

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Figure 2.- Appearance of HeLa cells at the toxicity ratings indicated in each photograph. (×100)

After the cups were scored, the cells in the first 2 cups in the direction of increasing contaminant concentration to receive a toxicity rating of 3 were checked for viable cells. This check was made by removing the contents of these cups, centrifuging at 500g for 10 minutes, and resuspending the sediment in 1.0 ml of fresh medium. This procedure was repeated once and the resuspended sediment was then placed in a sterile cup, capped with tape, and incubated for an additional 48 hours. If, at the end of 48 hours, there were no attached cells in these cups, the cells in these cups were considered dead and the toxicity rating of 3 was retained for these cups. If any of the cells in these cups appeared viable in fresh medium, the toxicity rating for that cup was changed to 2.

RESULTS AND DISCUSSION

The routine sterility and pleuropneumonia-like-organism checks showed bacterial contamination only very rarely and no evidence of pleuropneumonia-like organism. Those cultures found contaminated with bacteria were discarded immediately.

The results of a primary screening of forty-nine contaminants, which might occur in spacecraft, against HeLa cells in culture are shown in table II. The contaminants in this table are ranked in order of decreasing toxicity as defined by their cell toxicity index (CTI). This index was obtained by a summation of the toxicity ratings (average of the 2 cups in the duplicate series) for all the cups in the dilution series for each of the contaminants. In cases where the initial cup in the series did not contain 250 000 ppm of contaminant, because of insolubility at this concentration or because of lack of a sufficiently concentrated reagent solution (that is, methanal), it was assumed that this cup would receive a toxicity rating of 3. It was also assumed that after a toxicity rating of 0 had been obtained for 3 consecutive cups, in the direction of decreasing contaminant concentration, all subsequent cups would receive a toxicity rating of 0. Table III illustrates a typical data sheet used in recording the results of the contaminant dilution series.

Table II also contains data on the cytotoxic and lethal endpoints observed for each of the contaminants. The cytotoxic endpoint is the least contaminant concentration that contained cells exhibiting a definite morphological reaction to the contaminant. This condition occurred in the first cup with a toxicity rating of at least 1. The lethal endpoint was the lowest concentration of contaminant in which all of the cells were killed as shown by their failure to grow in fresh medium. This condition occurred in the first cup with a toxicity rating of 3. Other investigators (refs. 6 to 8) using similar screening methods have reported their data in terms of these parameters. Because of differences in the slopes of the various dose-response curves after 48 hours (see fig. 3, for example), one cannot rank the contaminants in terms of relative toxicities with regard to one of these parameters without disregarding the other. That is, in some cases, by using the fivefold dilution factor, the cytotoxic endpoint and lethal endpoint appear to occur at the

TABLE II.- TOXICITIES OF POSSIBLE SPACECRAFT CONTAMINANTS IN CELL CULTURE

Contaminant	Cell toxicity index	Lethal endpoint, ppm	Cytotoxic endpoint, ppm	Threshold limit value (ref. 5), ppm
2-Butenal (Crotanaldehyde)	39	0.005	0.0002	2 (tentative)
Propenal (Acrolein)	36	.03	.001	.1
Methanal (Formaldehyde)	31	.1	.03	5
Aminomethane	27	3.2	.1	10 (tentative)
2-Aminopropane	27	3.2	.1	5
1-Amino-2-methylpropane	27	3.2	.1	-----
1-Aminotoluene	26	3.2	.6	-----
2-Aminoethanol	24	16	.6	-----
1-Aminopropane	23	16	.6	-----
1-Aminopentane	23	16	.6	-----
Ethanal (Acetaldehyde)	23	16	.6	-----
Propanal	23	16	.6	-----
2,3-Butanedione	23	16	.1	-----
2-Methylpropanal	21	80	3.2	-----
2-Amino-2-methylpropane	19	80	16	-----
Ethanoic acid (Acetic acid)	19	80	16	10
Propanoic acid	19	80	16	-----
2-Methylpropanoic acid	19	80	16	-----
2-Methylpropenoic acid	19	80	16	-----
2-Aminobutane	18	80	80	-----
Dipropylamine	18	80	80	-----
Methanoic acid (Formic acid)	18	80	80	5 (tentative)
Dibutylamine	18	400	16	-----
Ethyl formate	18	400	16	100
Methyl formate	17	400	80	100
Butanoic acid	16	400	80	-----
Cyclohexanone	15	400	400	50
Methyl acetate	15	400	400	200
Cyclopentanone	15	2 000	80	-----
Ethyl acetate	15	2 000	80	400
1-Nitropropane	13	1 000	1 000	25
2-Butanone (Methyl-ethyl ketone)	13	2 000	400	200
3-Pentanone	13	2 000	400	-----
3-Heptanone	13	2 000	400	50 (tentative)
Cyclohexanol	13	2 000	400	50
2-Chlorethanol	12	10 000	400	-----
Nitromethane	12	10 000	100	100
2-Pentanol	11	10 000	2 000	-----
2-Propanone (Acetone)	10	10 000	400	1 000
2-Butanol	10	10 000	2 000	150 (tentative)
3-Pentanol	10	10 000	2 000	-----
2-Propenol	9	10 000	400	2 (skin)
1,4-Dioxane	9	10 000	10 000	100 (skin)
1-Butanol	7	10 000	2 000	100
1,2-Ethanediol	7	50 000	10 000	-----
1-Propanol	6	10 000	10 000	200 (tentative)
2-Propanol	5	50 000	10 000	200 (tentative)
Methanol	4	50 000	10 000	200
Ethanol	4	50 000	10 000	1 000

TABLE III.- TYPICAL SCORING CHART FOR CUP PANEL DILUTION SERIES

Contaminant	Scoring for contaminant in cup –											Cell toxicity index (CTI)
	1	2	3	4	5	6	7	8	9	10	Control	
1,4-Dioxane	3	3	3	0	0	0	0	0	0	0	0	9
2-Chloroethanol	3	3	3	2	1	0	0	0	0	0	0	12

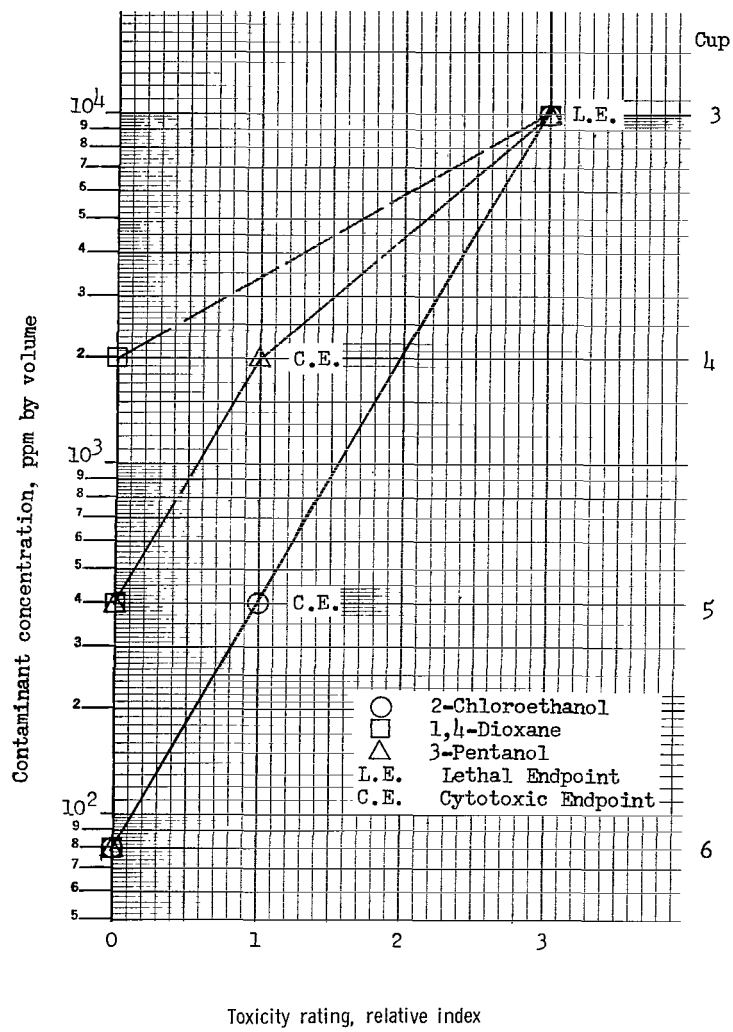

Figure 3.- Dose-response curves for 2-chloroethanol, 1,4-dioxane, and 3-pentanol after 48 hours.

same concentration (for example, 1,4-dioxane) whereas with other contaminants these two endpoints are separated by a relatively wide concentration differential (for example, 2-chloroethanol). The cell toxicity index takes both of these into account. This condition was of interest in this investigation because of the desire to rank the contaminants in terms of their relative toxicities to the primary screen. It should be noted that these endpoints are endpoints only in terms of a fivefold dilution series.

No apparent change could be determined in the cells in any of the control cups or control series. It is interesting to note from the data presented in table II that there seems to be a general correlation between the chemical class of the contaminant and its toxicity to cells in culture. That is, in order of decreasing toxicity the contaminants might be ranked as follows: unsaturated aldehydes, amines, aldehydes, acids, ketones, and alcohols.

Figure 4 illustrates the results obtained by relating the toxicity of these contaminants in cell culture, as defined by their cell toxicity index, and their toxicity in man, as shown by previously established threshold limit values (ref. 5). The data points shown represent the various compounds with known threshold limit values and relate these threshold limit values to the cell toxicity indices obtained for these compounds in cell culture. Although the correlation is not absolute, it is apparent that the trend is toward a direct relationship between toxicity in man and toxicity in cell culture.

No attempt was made to analyze these data statistically. However, the results of preliminary studies completed 6 months previous to the work reported in this paper agree very closely with the results of this work. Table IV compares the results obtained in the earlier studies with the results shown in table II. It would therefore seem that the method is at least moderately reproducible. On the basis of this apparent reproducibility and the general correlation of these data with toxicity data in man, it is concluded that cell culture can serve as a primary toxicity screen for compounds being investigated with regard to man.

The effects of synergism or antagonism between contaminants were not investigated in these experiments. However, the short time required for the development of observable results (48 hours) would make it possible to study these problems easily. It is also possible that a lower concentration of contaminant applied to the cell cultures for longer periods of time would yield morphological indications of toxicity. However, the objective of these experiments was not to define the level of toxicity of these contaminants to cells in culture but to attempt to determine a preliminary indication of the relative toxicities of these compounds to a living system.

In this paper, data are reported on the toxicity of the contaminants listed to only one cell line. It would be desirable to repeat these experiments in several cell lines, including some derived from normal human tissue, in order to obtain responses from

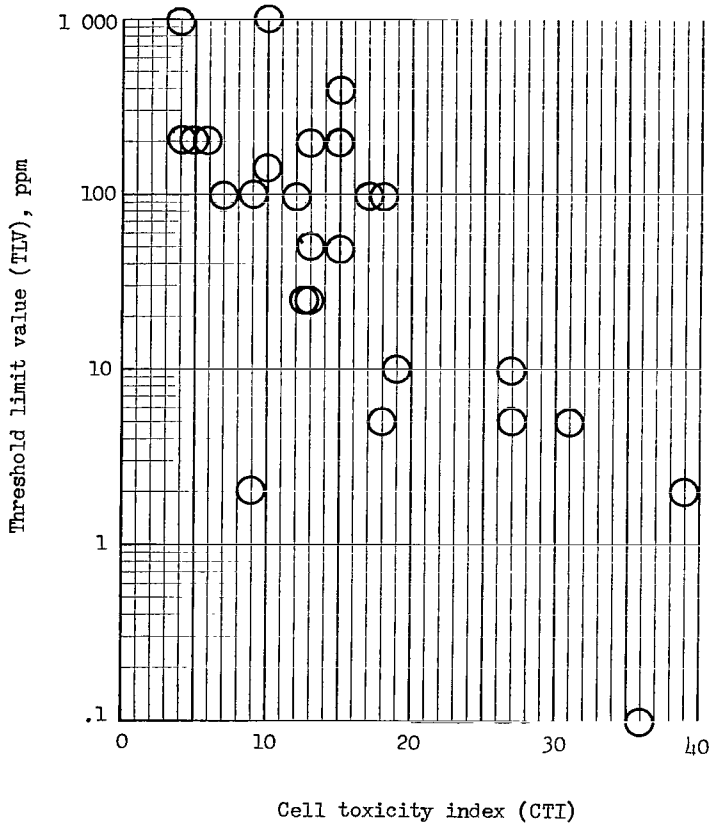

Figure 4.- Relationship between toxicity in cell culture and in man.

different cell types to the various contaminants. Also, since diploid cell strains are generally more sensitive to changes in the environment and do not exhibit as much variation as permanent cell strains (ref. 11), it would be interesting to investigate the effects of these contaminants in a human-derived diploid cell strain.

The primary problems involved with this investigation are: (1) the solubility of the contaminants in the medium, (2) the reactions of the contaminants with the medium, and (3) the extrapolation of the data obtained in cell culture to data applicable to man. The detection of the difference between the cytotoxic and lethal endpoints for certain compounds must be further studied. The contaminants investigated in this report are only those which were soluble in the medium at a level of at least 5 percent (by volume). With suitable analytical support to determine the exact amount of contaminant under consideration, it would be easily possible to screen many other compounds that are only slightly

TABLE IV.- COMPARISON OF CELL TOXICITY INDICES DERIVED IN EXPERIMENTS SEPARATED BY 6 MONTHS

Contaminant	Cell toxicity index from –	
	Preliminary tests	Present tests*
Methanal	30	31
2-Methylpropanal	21	21
Ethanoic acid	20	19
Propionic acid	21	19
Methanoic acid	18	18
Butanoic acid	15	16
Methyl acetate	15	15
Cyclohexanol	14	13
2-Butanone	16	13
2-Propanone	6	10
2-Butanol	9	10
1-Propanol	7	6
Ethanol	7	4
Methanol	7	4

*Conducted 6 months after preliminary tests.

soluble in the medium. There are a multitude of reactions that could result between the contaminants and the medium. In future investigations it would seem appropriate to simplify the medium as much as possible.

CONCLUDING REMARKS

Forty-nine compounds which might occur as contaminants in a manned spacecraft atmosphere were screened for their relative toxicities to HeLa cells in culture. These contaminants are those expected to evolve from both the man and the spacecraft materials. The data were rapidly acquired, seem to be reproducible, and were relatively inexpensive to obtain. This technique provides a good first approximation of those substances that would be expected to be most objectionable to a living system. Thus, this technique appears to be of value as a rapid method for screening a long list of contaminants for those which should receive priority in further toxicological studies. A tentative observation based on the data presented in this paper is that the various chemical classes of contaminants might be ranked in order of decreasing toxicity to cells in culture as follows: unsaturated aldehydes, amines, aldehydes, acids, ketones, and alcohols.

Langley Research Center,
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