Inquiry into the Limits of Biological Effects of Chemical Compounds in Tissue Culture, I

Low Dose Effects of Mercuric Chloride

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Mercurichloride, Low-Dose Effect, Drug-Effect Relation, Serial Dilution, Tissue Culture

Experiments were designed to investigate the low-dose side of the so-called dose-effect relation of mercuric chloride on the proliferation of a murine lymphoblastic cell strain (MB VIa). Three concentration ranges, from 0.9×10^{-5} M to 0.9×10^{-18} M, from 0.9×10^{-12} M to 0.9×10^{-25} M, and from 0.9×10^{-15} M to 0.9×10^{-21} M, in which the concentration decreased stepwise by a factor 10, were studied in 61, 74 and 58 experimental repetitions, respectively.

In the first range, the concentrations 0.9×10^{-5} M and 0.9×10^{-6} M $HgCl_2$ proved to be definitely toxic as was expected. However, also 0.9×10^{-16} M and 0.9×10^{-17} M appeared to be growth-inhibiting, the latter results being confirmed in the second and the third concentration ranges. These differences in cell growth were statistically significant.

Introduction

Among the quantitative studies in cell and tissue culture, the toxicity titration method by serial dilutions is one of the most commonly used. The response of the cells or tissues to the tested substances frequently follows a definite mathematical relationship, which shows a sigmoidal nature: its major part being a straight line with inflexion points at relatively high and low doses, respectively (see, for instance [1]). However, data from literature revealed that many discrepancies from the theory were found experimentally which generally remained undiscussed.

First there is the generally accepted idea, also mentioned by Paul, that "where experimental observations can fit into a linear relationship of this kind (dose-response curve) it is obvious that fewer replicates at a given concentration are required, relationship of the investigated system is likely to be since the general trend of the line acts as a test for the accuracy of the results".

Though the argument may be convincing it implies a tendency to adapt the results to a basically hypothetical relationship between substance and cells (substratum), for it encourages the experimenter to regard deviations from the supposed curve as outliers. In such a way a hitherto unknown property or overseen.

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A second rather common trait is that the agent-substrate relation is more attentively investigated on the side of the moderate and stronger effects, *cf.* higher doses, than on the side of the diminishing effects on the dose-response curve. The latter can be easily extrapolated towards the zero level *i. e.* the data obtained from the controls. Only few investigators really go as far as to affirm this experimentally in their papers.

Yet, where papers do mention data on the low-dose side of the curve, *i. e.* values approaching the second inflexion point, it becomes clear that further investigations would not be altogether superfluous. For example Nayak and Mashelkar [2] working with copper, manganese and molybdenum on L-strain cells observed rather irregular dose-response relations.

Hulliger *et al.* [3] found an unusual effect for $ZnCl_2$ on the growth of rabbit fibroblasts in culture, the salt being less toxic at the 10^{-5} M level than at 10^{-3} M or 10^{-7} M levels.

Schöpf *et al.* [4] observed that the highest transformation rates of lymphocytes brought about by $HgCl_2$, were caused by 3.7×10^{-5} M, whereas 3.0×10^{-5} M and 6.6×10^{-5} M were markedly less effective. Recently, Roessner and Bencko [5] reported a study on the effect of Na_2SeO_3 on tissue culture cells. Apart from morphological observations they found that in a concentration between 10^{-4} M and 10^{-11} M, cell proliferation was inhibited by 10^{-8} M and 10^{-9} M whereas 10^{-6} M, 10^{-7} M and 10^{-10} M

were not equally toxic. Unexpectedly, 10^{-11} M showed a stimulatory influence, exceeding the control values.

Since the drugs, used by Schöpf *et al.*[4] and Roessner and Bencko[5] probably are no micronutrients, their modes of action at lower doses cannot be simply ascribed to a certain physiological (catalytic) role (apart from their toxic effect at higher doses). Especially when too much confidence has been placed beforehand in the idea that beyond a certain concentration a horizontal course in the drug-effect relation is obtained, the possibility exists that valuable information about the agent-organism relationship may remain undiscovered. At very low doses such a confidence may easily lead the investigator either to regard most deviations as unavoidable biological deviations from the relationship that "really exists" (*i. e.* the biased horizontal course), or to make uncritical extrapolation of too few determined points.

As to the introduction of systematic errors in a given experiment (for example pipetting errors, instrument-reading errors, weighing errors) one can cope with these irreliabilities by massive repetition of the experiment. Rigorous randomisation of all experimental human handling in regard to the range of tested concentrations is likewise needed in combination with the above-mentioned repetition to meet arguments of this kind. In a variety of papers, many of which have recently reviewed by Pedersen[6], the occurrence of reproducible statistically significant deviations from the expected horizontal section of the dose-response curve at very low dose levels was reported. Although the magnitude of these deviations is only rarely very impressive, their reproducibility implies that they cannot be considered random.

In the light of the above a renewed careful study of the diminishing effects of decreasing concentrations of biologically active substances seemed to be worthwhile. In the present study the following question was posed: In a long range of serial dilutions does the dose-response curve assume a flat course at very low doses, or are there, indeed, statistically significant and reproducible deviations as reported by other investigators? Therefore, the following statistical working-hypothesis (H_0) could be put forward: In a (sufficiently long) range of serial dilutions the dose-response curve gradually tends towards a definite horizontal (zero) level.

As a test agent mercuric chloride was chosen because it is an easily soluble compound of this well-known poisonous metal. It was supposed to reveal a simple dose-effect relation, as no nutrient or catalytic functions in normal biological systems have been found up till now.

Lymphoid cells were used as substrate because – according to Schöpf *et al.*[4] – they seem to be very sensitive to mercury.

Material and Methods

Preparation of successive dilutions of mercuric chloride

A 10% (w/v) solution of mercuric chloride was prepared by dissolving 10 g of $HgCl_2$ (Merck p.a.) in 100 ml water of 80 °C. This solution was pipetted into a 200 ml Erlenmeyer flask and handshaken during 4 min. 5 ml of this solution (10^{-1} concentration) was added to 45 ml water in another Erlenmeyer flask of 200 ml which was shaken in the same way. So this 1:10 dilution resulted in a 10^{-2} concentration 1% w/v). Successive 10^{-3}, 10^{-4}, 10^{-5} concentrations etc. were made in exactly the same way. Each pipette was used only once and each flask was chosen at random. Two controls of mere aqua bidest were included in each series. These were not shaken. Glassware was cleaned and sterilised as usual in tissue-culture practice. The water used was glass-double distilled and sterilised by ultrafiltration through millipore filters of 0.22 μm pore size.

To these dilutions equal amounts of double strength TC 199, a tissue culture medium composed by Morgan, Morton and Parker[7], was added after similar ultrafiltration. As a result of this mixing the concentration of mercuric chloride and TC 199 decreased by a factor 0.5, making the latter concentration appropriate for cell growth. This series of dilutions was used as test media for the propagation of cell cultures.

Culture method

A lymphoblastic cell line of murine origin explanted from a lymphosarcoma in 1954 is serially propagated in our Department using TC 199 as culture medium, supplemented with 10% human serum and antibiotics (50 i. u. penicillin, 50 μg streptomycin and 50 μg Kanamycin per ml). This cell line called MB VIa[8] grows in stationary suspension cultures at 36.5 °C in small Erlenmeyer flasks. The spherical MB VIa cells have a diameter varying between ± 11 μm and ± 40 μm. Medium is changed 2 times weekly and the harvested cell suspensions

pooled. Such pooled suspensions were used for each experiment.

Incubation of the culture tubes

Test tubes of Duran glass (Schott & Gen.) having an internal diameter of 9 mm and a length of 130 mm were marked and placed in racks using random numbers to allot their location in it. Into each tube 1 ml of cell suspension, adjusted to 2000 cells/ml was inoculated by means of an automatic pipette (Cornwall) at a constant sequence to effectuate the mentioned randomisation. Next the tubes were provided with 1 ml mercuric chloride dilutions (in TC 199) in a sequence differing from that used for inoculation. For each dilution 5 tubes were used. Thus all the tubes had a final volume of 2 ml. When, for example, a 0.5×10^{-5} sublimate/TC 199 solution was used, each of the 5 test tubes had a concentration of 1000 cells/ml and 0.25×10^{-5} g $HgCl_2$ per ml or 0.9×10^{-5} M $HgCl_2$. Corresponding to the degree of dilution, these cultures were typed Hg5, those containing 0.9×10^{-6} M $HgCl_2$ were typed Hg6 etc. The tubes were closed with silicone rubber stoppers, placed in racks and incubated during 90 hours at 36.5 °C. Each tube was randomly placed into the incubator.

Cell counting

After incubation the racks were cooled at room temperature (±20 °C) for 30 min to stop cell growth and division.

Then, in random order, the 5 tubes containing the same mercuric chloride dilution were shaken on a Vortex mixer during some 4 sec to obtain a unicellular suspension. Next their contents were mixed and diluted with Isoton fluid (1:7), (Coulter Counter Electronics, Dunstable, Bedfordshire). The solutions obtained in this way were counted without delay using a Coulter Counter model F, calibrated with latex particles. The settings of this apparatus were chosen in order to count the cells with a diameter ≥ 16 μm. Four repeated counts were made and averaged. Thus every experiment yielded 16 mean counts (14 dilution levels and 2 controls). (In a latter 3rd series of experiments the number of the tested dilutions was changed from 14 to 7, to which only one control was added, resulting in 8 mean counts.)

Statistical procedures

Three statistical tests were used:

1. Mean cell counts for each concentration level over the series of experiments were calculated with their 95.5% probability range (confidence interval) and plotted by computer. The same was done after reducing all data of each experiment to percentual deviations from the mean of that experiment.

2. The Friedman (l. c. 9, G. J. Smit, personal communication) test which is sensitive to fluctuations in repeated series of observations. This is a non-parametric test. The null-hypothesis in this case is that differences between the countings in the various dilutions are fortuitous.

3. Statistical significance of differences between pairs of dilutions was tested by the Student's and Wilcoxon's [9] methods. These tests are parametric and non-parametric respectively.

In all tests a level of significance of 0.05 was regarded as decisive for either rejection or acceptance of the null-hypothesis. Other levels are sometimes mentioned for reasons of curiosity and extra information.

Results

First, eight 1:10 dilution series were tested 61 times covering the concentration range from 0.9×10^{-5} M down to 0.9×10^{-18} M. So each dilution series was used several times, and 61 data for each of the 16 treatments were registered.

When the mean results of each of the 16 treatments (14 dilution steps and 2 controls) were plotted with their 95.5% confidence interval regions ($2\,s/n^{1/2}$, in which s = standard error of the sample mean and n = number of experiments), only Hg5 appeared to be definitely toxic. In addition, however, two other observations were made: a. The 95.5% confidence intervals were rather wide, indicating flat frequency distributions of the countings, and b. the deviations of Hg6, Hg16 and Hg17 were relatively large, which was the more surprising because of the 61-fold repetition of the experiment.

To find out whether all 16 treatment groups, each of 61 numbers, could be regarded as samples of one and the same population the Friedman test was applied [9], G. J. Smit, personal communication. This resulted in the unexpected conclusion that the suggested nullhypothesis (H_0) had to be rejected (see Table I).

However, this outcome was greatly influenced by the presence of the low countings for Hg5.

Hence, the test was repeated disregarding Hg5. Again the outcome was that H_0 had to be rejected.

Now Hg6, being the second greatest deviation, was omitted. The necessity for rejection of H_0 was indicated again.

Table I. Table of results of Friedman tests.

	Test statistic	No. of expts.	No. of treatments	Extract from χ^2 table $\chi^2_{0.90}$	$\chi^2_{0.95}$	$\chi^2_{0.99}$
1st series, all treatments	167	61	16	22	25	31
1st series, without Hg_5	38	61	15	21	24	30
1st series, without Hg_5, Hg_6	24	61	14	20	21	26
1st series, without Hg_5, Hg_6, Hg_{16}	14	61	13	19	21	26
2nd series, all treatments	38	74	16	22	25	31
3rd series, all treatments	10	58	8	12	14	18
1st+2nd series	19	135	8	12	14	18
2nd+3rd series	9	132	8	12	14	18
1st+2nd+3rd series	15	193	5	8	9	13

From this evaluation it became clear that Hg5, Hg6 and likely also Hg16 and Hg17 brought about deviations which for all but Hg5 were masked by overlying variations of unknown origin.

One way to cope with such masking variations consists of expressing all the results of one experiment as percentual deviations of the overall mean of that experiment (J. Strackee, personal communication). Each treatment was therefore expressed as the mean of (in this case) 61 percentual deviations.

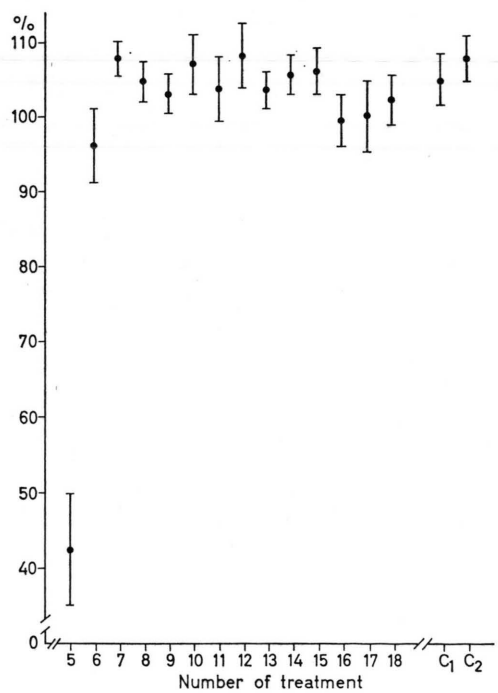

Fig. 1. Results of the 16 treatments of the 1st series, expressed as means of the 61 percentual deviations. The values were obtained by expressing each of the 16 mean cell counts of one experiment as a percentage of the overall mean cell count of that experiment. Vertical bars represent the 95.5% confidence intervals of the means.

Now these mean percentages were plotted (see Fig. 1).

It is this presentation of the results that clearly demonstrates the real differences between the effects of the treatments already indicated by the outcome of the Friedman test: Hg5, Hg6, Hg16 and Hg17 are different from most other treatments. That the two controls C_1 and C_2 lie higher than the 100% level is due to the fact the percentages are computed in respect of the mean of all treatments, including Hg5 and the other low ones.

Proceeding from this presentation of the results, pairs of treatments were chosen to be tested, using Student's method for differences of paired observations and Wilcoxon's symmetry test [9] (see Table II).

In this way additional information was gained which completed the information obtained from the Friedman tests and the plot of reduced (percentaged) data [10].

In a second series of 74 repetitions the range from Hg12 to Hg25 was chosen; the first part, Hg12 to Hg18, to investigate the experimental reproducibility of the unexpected results, the second part, *viz.* Hg19 to Hg25 to scrutinize the curve for either the zero-part or possible effects of still lower concentrations. Again two controls were included. When the mean values of the absolute data were plotted no deviations from the supposed flat curve were found. However, applying the Friedman test to our null-hypothesis, it became clear that the mean values of the 16 treatments did not belong to the same population (see Table I). Plotting the data in percentages (Fig. 2) showed that the trend in the range from Hg12 to Hg18 closely resembled the corresponding section of the curve of the first series.

The Student and Wilcoxon methods reveal statistical differences between the following pairs of

Table II. Table of Student's and Wilcoxon's test. Two-sided tail probabilities. $P<0.001$, fat; $P<0.010$, italic; $P<0.050$, underlined; $P>0.050$, not underlined.

Pairs of treatments	Series 1		Series 2		Series 3		Series 1+2		Series 2+3		Series 1+2+3	
	Stud.	Wilc.	Stud.	Wilc.	Stud.	Wilc.	Stud.	Wilc.	Stud.	Wilc.	Stud.	Wilc.
C_1-C_2	0.526	0.237	0.168	0.280	—	—	—	—	—	—	—	—
$Hg_{16}-C_1$	0.013	0.021	0.742	0.511	0.518	0.487	*0.002*	*0.001*	—	—	—	—
−6	0.276	0.417	—	—	—	—	—	—	—	—	—	—
−7	**0.001**	**0.001**	—	—	—	—	—	—	—	—	—	—
−10	*0.007*	*0.004*	—	—	—	—	—	—	—	—	—	—
$-C_2$	*0.002*	**0.001**	0.304	0.432	—	—	—	—	—	—	—	—
−12	*0.003*	*0.003*	0.317	0.480	—	—	*0.002*	*0.007*	—	—	—	—
−14	*0.007*	*0.005*	0.031	0.051	—	—	**0.001**	**0.001**	—	—	—	—
−15	*0.008*	*0.007*	0.016	0.052	0.660	0.592	**0.001**	**0.001**	0.021	0.075	**0.001**	*0.002*
−18	0.280	0.094	**0.001**	*0.002*	0.728	0.938	*0.008*	**0.001**	*0.009*	0.012	0.018	*0.002*
$Hg_{17}-C_1$	0.052	0.306	0.845	0.846	0.051	0.112	0.014	0.057	0.327	0.239	*0.004*	0.017
$-C_2$	*0.007*	0.016	0.105	0.122	—	—	—	—	—	—	—	—
−15	0.022	0.022	0.198	0.435	0.077	0.055	*0.010*	0.264	0.038	0.067	*0.004*	*0.005*
−16	0.708	0.804	0.206	0.282	0.148	0.328	0.761	0.303	0.736	0.826	0.900	0.624
−18	0.103	0.144	0.033	0.020	0.366	0.510	0.013	*0.010*	0.026	0.025	*0.008*	*0.009*

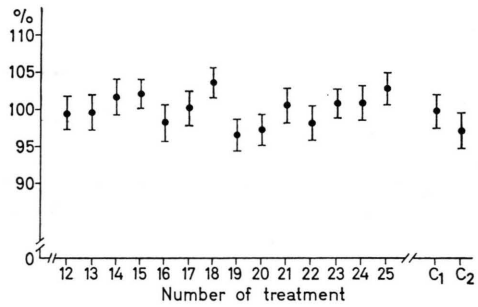

Fig. 2. Results of the second series of 74 experiments. The values were obtained as in Fig. 1. Vertical bars indicate the 95.5% confidence regions of the means.

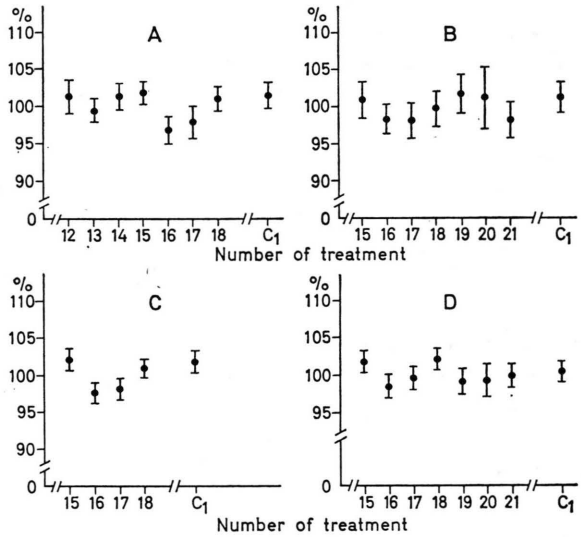

Fig. 3 A. Mean percentages of the treatment results in the overlapping area of the 1st and the 2nd series. Each value is the average of 135 percentages (see Figs 1 and 2).
Fig. 3 B. Mean percentages of the 3rd series of experiments. Each value is the average of 58 percentages, expressed in the same way as Figs 1 and 2.
Fig. 3 C. Mean percentages of the treatment results in the overlapping area of the 1st, 2nd and 3rd series of experiments. Each mean value comprises 193 experimental data.
Fig. 3 D. Mean percentages of the treatment results in the overlapping area of the 2nd and 3rd series of experiments. Each mean value comprises 132 experimental data.
Fig. 3, A−D: Vertical bars indicate the 95.5% confidence regions.

treatments: Hg19 and Hg23, Hg19 and Hg24, Hg19 and Hg25, Hg20 and Hg25 (see Table II). Although this second series in the range of Hg12 to Hg18 may be considered as reproducible with respect to the corresponding part of the first series, a more exact way to confirm this by statistical calculation is found in compiling the data for the corresponding (overlapping) areas of the two dilution ranges. This is shown in Fig. 3 A. See also Table I, 1st + 2nd series. Moreover, the results of pairwise testing of the treatments by Student's and Wilcoxon's methods as before gave full support to the observations mentioned above. The *P*-values for the pairs tested decreased when combining the 1st and the 2nd series (Table II).

The object of a third series was an attempt to reproduce the most characteristic traits of the curves: the dip between Hg15 and Hg18 in the 1st and in the 2nd series of experiments and also the dip between Hg18 and Hg21 in the 2nd series. Consequently, a range from Hg15 to Hg21 was chosen.

Neither the absolute cell countings, nor their percentual reduction showed any statistically significant differences. However, the data obtained in the third series clearly support the view that prolonged repetition of the experiment could lead to reduction of the standard deviations of the respective means of the treatments. Its curve (Fig. 3 B) roughly shows the same dip at Hg16 and Hg17, as in the 1st and 2nd series.

One possible way to achieve such an indication lies in adding the results obtained previously to those of the third series; *i. e.* to evaluate all 193 experimental repetitions of the 5 overlapping treatments: C_1, Hg15, Hg16, Hg17 and Hg18. The result is given in Fig. 3 C. The Friedman test yielded G = 15 (see Table I). For *P*-values obtained with Student's and Wilcoton's test see Table II. Compiling only the data of the 2nd and 3rd series resulted in Fig. 3 D; see also Tables.

So this part of the third series, although not conclusive in itself, cannot be considered to be in disagreement with the first and the second series. The high-dilution part of the third series, on the other hand, differs from the corresponding part of the second series. In the latter, Hg18 differed significantly from both controls and also from Hg19 and Hg20 (Table II). In the third series, however, no significant deviation was observed at all. In contrast to the findings in the region Hg15 to Hg18, the trend found in this part of the curve seems to be opposed to that of the corresponding part of the curve of the second series. Hence, in order to arrive at satisfactory statements about the action of the treatments Hg19, Hg20 etc. further research is needed.

Finally, Fig. 4 shows the results of the reduced (percentaged) data of the three series in a total of 193 repetitions of the experiment, brought together according to their respective treatment numbers. Treatment Hg5 is left out of the figure because it lies far below the abscissa (see also Fig. 1).

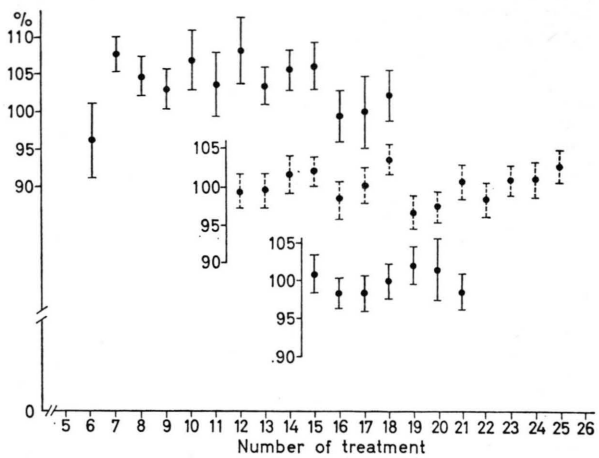

Fig. 4. Treatment results of each of the three series brought together in one representation. Separate ordinates: percentages with their 95.5% confidence regions.

Discussion

It appeared that the surprising growth-inhibiting effect of Hg16 and Hg17, found in the first series could be experimentally reproduced in the two following series, in contrast to some other traits of the successive series. Within the first as well as the second series this effect of Hg16 and Hg17 was already found to be of statistical significance. After establishing these findings, a discussion of the low-dose problem should follow in respect of theoretical considerations about the possible mode of action of the treatments.

One of the problems in these experiments is that one has to account for the concentration of $HgCl_2$ resulting from micropollution of the used chemicals, glassware, water etc. As the maximum limits of impurities in chemicals of analytical grade amount to about 10^{-9} to 10^{-10}, from a theoretical point of view one never can exceed these limits when diluting a substance in a medium composed of such chemicals. In this case, theoretically, the dose-effect curve would show a flat and horizontal part when passing the concentration level of the impurities. However, such a type of curve could not be demonstrated.

Thus, apart from the above mentioned impurities, one has to make allowance for a second theoretically unpassable threshold, namely that of the particle-bound (*i. e.* molecule- or ion-bound) action which is responsible for the effect exerted by a chemically defined substance.

Although toxic effects in the one molecule/one cell ratio are known in the literature [11], one may expect on hypothetical grounds that prolonged dilution, of any substance whatsoever would result in a dose-effect curve that beyond a certain concentration leads to a flat part of the curve. As our experimental data were unable to demonstrate such a fading in the relationship between particle concentration and

its exerted effect, this theoretical consideration (and the former mentioned) is possibly aprioristic.

Unger [12] suggested a physicochemical model pointing to the modifications of the structure of the diluting medium (water); it might be such a factor instead of the direct chemical action of the dissolved substance which brings about the observed effects. According to theories concerning information processing (information theory) this action could be transferred from one dilution to the next.

As the results of the statistical methods used are in close agreement, there is no doubt that the interpretation of the experimental results is adequate. This means that there are actually some non-fortuitous differences in action between the treatments Hg16 and Hg17 and many others. The many repetitions from which the above mentioned conclusions are derived, together build up an exceptionally strong foundation for these conclusions.

Apart from endogenous fluctuations in cell growth and mean cell volume [13–15], external factors like barometric pressure, solar radiation, electromagnetic wave patterns, seasonal influences, cannot be excluded [16–21]. However, in the reported work possible actions of external origin could only have modified the cell propagation of all individual treatments to the same extent and are not likely to account for the differences between the treatments, because they grow simultaneously in each repetition of the experiment. This renders it impossible to regard the observed deviations of our null-hypothesis as results of any random variations or of systematic experimental errors. Nothing else than the tested treatments could be responsible for the reported results. In this regard, a number of the publications reviewed by Pedersen [6] are in close agreement with our results.

However, the many questions arising as to the mode of action of the tested treatments can not be answered as yet. On this point no conclusions can be derived from our data as our experiments were not designed to investigate these questions. The substantial indication towards some as yet unconceived phenomena needs further study.

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