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Analyses of a Toxic Factor, Lethal to Paramecium Present in Non-Glass-Distilled Water. (25061)

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While working on the effect of proteolytic enzymes on *Paramecium aurelia* (1) we became aware of an unknown substance in our distilled water which caused the death of this protozoan within 20-30 minutes. The same water, after redistillation in Pyrex glass (to be referred to as doubly glass-distilled), was no longer toxic for these organisms. The toxicity pattern is as follows: First, the ciliate's locomotion is affected, causing it to move erratically in all directions and to spin around on its long axis. Body deformation then sets in usually accompanied by ectoplasmic extrusion, or "blistering" (Fig. 1). Finally, the afflicted organism slows down and succumbs. After this, some degree of disintegration of the cytoplasm of the dead organism occurs (Fig. 2) seldom involving the nucleus. According to Heilbrunn (2) toxic action of distilled water can be attributed in certain instances to presence of poisonous impurities which may presumably represent contamination products from the conventional types of metallic distillation apparatus. It is also known (*loc. cit.*) that a trace of calcium is capable of neutralizing the action of these substances. But the toxic agent or agents were not identified, nor was the nature of their action sufficiently elucidated. The purpose of this study was to use *Paramecium* as the test organism in an attempt to analyze the toxic factor or factors in our distilled water.

Materials and methods. A kappaless stock of *Paramecium aurelia*† was cultured in a medium containing boiled oats. The culture reached peak growth in about 2 weeks, stayed luxuriant for a few more days, then rapidly declined. Doubly glass-distilled water‡ was used exclusively for culturing, making solutions as well as washing glassware. The experimental procedure was as follows: The Paramecia were transferred with a micropipette from an actively growing culture at its peak growth to a depression slide (3″ x 1¾″) with a central 1⅜″ diam. depression. Two drops (40 drops equal approximately 1 ml) of culture medium, containing no less than 100 paramecia, were added to 1½ ml of the test solution. Observations were made at room temperature with both stereoscopic and compound microscopes. A moisture-saturated Petri dish was utilized for maintenance of test cultures for lengthy periods of observation. Stock solutions (0.001 M) were prepared from which appropriate dilutions or combinations were made. Routine pH determinations were made with a Beckman pH meter on all test solutions. 35 mm Kodachrome slides were made with the aid of an AO Spencer 20X dark phase contrast objective. The 3 slides used as illustrations were printed from negatives taken from the original Kodachrome transparencies. A threshold concentration is specified as one which most closely duplicates the toxicity of the distilled water in question, *i.e.*, death of all or nearly all paramecia in 20-30 minutes, preceded by morphological

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† Stock No. 51, courtesy of Dr. T. M. Sonneborn.

‡ Courtesy of Dept. of Biochemistry, Stritch School of Medicine.

changes comparable to those described above (cf. Fig. 3 with Fig. 1 and 2). It is not a minimum lethal concentration as may be normally denoted by the term "threshold." The reason for this is clear when the results, especially those in connection with the capability of calcium to neutralize the toxic action, are considered. The results presented below represent reproducible observations through repeated trials for each experiment. Three series of experiments were carried out: (a) to determine if change in pH is a factor in the observed toxicity; (b) to duplicate the toxicity of our distilled water by trace quantities of several salts of heavy metals; and (c) to ascertain the role of calcium in counteracting or neutralizing the toxic action of distilled water and salt solutions.

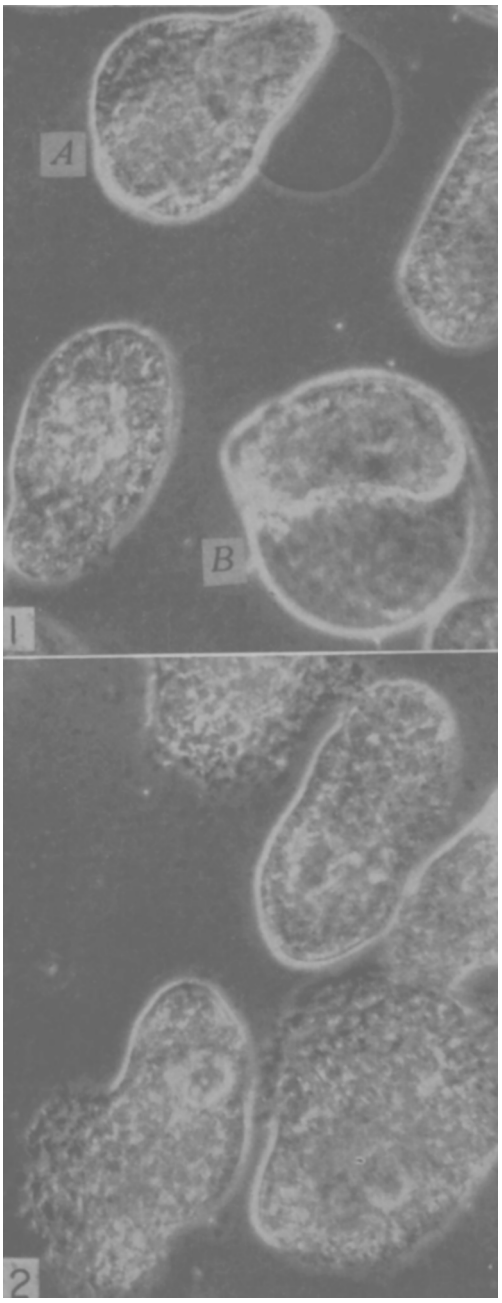

FIG. 1. Paramecia showing varying degrees of body deformation after 16 min. in "toxic" distilled water. Note: 1) typical ectoplasmic "blister" (A); 2) a similar blister filled with endoplasmic granules as a result of pre-mature bursting of plasma membrane (B). Magnification: 250×.

FIG. 2. Dead paramecia undergoing varying degrees of disintegration after 22 min. in "toxic" distilled water. Note intact nuclei in each. Magnification: 250×.

Results. Role of pH. The pH of our distilled water was 5.6, with a normal range of 5.4 to 6.0. After neutralizing this distilled water with $LiCO_3$ or $NaHCO_3$ to pH 7.0, there was no change in its toxicity for *Paramecium aurelia.* To indicate whether $LiCO_3$ and $NaHCO_3$ themselves were toxic to *Paramecium,* each of these salts was

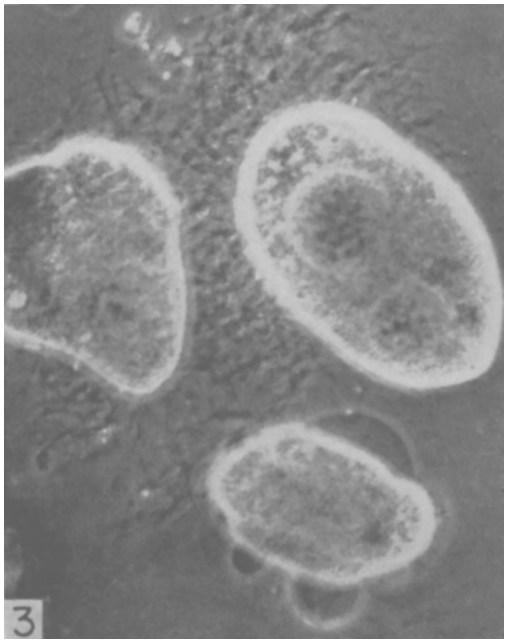

FIG. 3. Three paramecia after 10 min. in 0.000002 M $CuSO_4$ revealing similar toxic symptoms to those caused by the distilled water (one of them showing 2 macro-nuclei, possibly in late anaphase or early telophase). Magnification: 250×.

TABLE I. Threshold Concentrations (M) of 5 Salts Comparable to Toxicity Level Found in Non-Glass-Distilled Water and Lethal to *Paramecium aurelia* in 20-30 Minutes. The chemicals are listed in order of their relative degree of toxicity.

Chemicals	Threshold
$CuCl_2 \cdot 2H_2O$	10^{-6}
$CuSO_4 \cdot 5H_2O$	20^{-6}
$Cu(NO_3)_2 \cdot 3H_2O$	20^{-6}
$Zn(C_2H_3O_2)_2$	10^{-4}
$Pb(C_2H_3O_2)_2$	25^{-4}

dissolved in doubly glass-distilled water in amounts sufficient to bring the pH to 8.9. In both instances no deleterious effect on this organism was observed. These observations coupled with the fact that the small amounts of the salts employed in the experiments brought negligible alteration, if any, to the pH of the distilled water provide sufficient evidence that pH is not a factor in the toxicity phenomenon under consideration.

Duplication of distilled water toxicity by solutions of copper, zinc and lead salts. Solutions of copper chloride, copper sulfate and copper nitrate, and zinc and lead acetate were tested. Observations were made on the toxicity effects of each of these solutions beginning with concentrations of 0.001 M and decreasing to a level at which toxicity symptoms approximated those obtained with the distilled water. The results are summarized in Table I. Approximately 1/100 the threshold concentration rendered these solutions non-toxic.

Role of $CaCl_2$ in neutralizing toxic factor. It was found necessary to determine first the concentration at which $CaCl_2$ itself is toxic to *Paramecium*. A concentration of 0.05 M $CaCl_2$ was lethal, causing death in 10 minutes with explosion of trichocysts. 0.035 M $CaCl_2$ approximated the toxicity level of our distilled water, while 0.02 M proved not only non-toxic by itself, but capable of neutralizing the toxicity of the distilled water. Paramecia lived in this "calciumized" water as well as they did in doubly glass-distilled water. The protection so rendered by calcium varies directly with concentrations of this salt. For instance, with 0.01 M $CaCl_2$ protection lasted only a few hours as compared to 2-3 days with a concentration of 0.015 M. Thus, there seems to be a quantitative relationship between concentration of $CaCl_2$ and extent of protection obtained. Next, we attempted to neutralize by means of $CaCl_2$ the toxicity of the aforementioned salts at their threshold concentrations (Table I). The results reveal a significance of both a quantitative and qualitative nature. Complete neutralization of the toxicity of 0.000001 M cupric chloride (threshold) was obtained with 0.015 M $CaCl_2$. We found this concentration of $CaCl_2$ to be critical, for either lowering or raising it modified the results sharply. For example, within the 0.006-0.013 M range paramecia lived up to a few hours but the presence of 0.017-0.025 M $CaCl_2$ in 0.000001 M $CuCl_2$ caused a slight enhancement of toxicity as evidenced by the fact that death of the organisms occurred sooner here than in 0.000001 M $CuCl_2$ alone. The threshold concentration of lead acetate, 0.0025 M, can be likewise completely neutralized with 0.015 M $CaCl_2$.

In sharp contrast to these results, none of the remaining 3 toxic salts, cupric sulfate, cupric nitrate and copper acetate, was found to be neutralizable by $CaCl_2$. The presence of $CaCl_2$ above 0.013 made these copper salts even more toxic to paramecia, confirming a similar phenomenon with $CuCl_2$ mentioned above. It was imperative to determine whether or not 0.015 M $CaCl_2$, while unable to neutralize the sulfate, nitrate or acetate of copper at their threshold concentrations, might nevertheless neutralize these compounds at lower concentrations. At 1/20 the threshold concentration of both cupric sulfate and cupric nitrate and 1/5 the threshold concentration of zinc acetate, the calcium salt still brought no neutralization. At these subthreshold concentrations, toxicity symptoms were much milder, and death of the organisms came after many hours of exposure. Also the paramecia were little affected in their movement, and showed less "blistering" and body deformation, as well as no bursting or disintegration of the dead organisms.

An analysis of the reasons for the contrasting behavior of cupric chloride and cupric sulfate toward $CaCl_2$ was accomplished by introducing equal molar strength of NaCl to the cupric sulfate and reciprocally Na_2SO_4 to the cupric chloride, assuming that all 4 salts are

about equally ionized. Addition of $CaCl_2$ to the 2 now ionicly equalized solutions should constitute a crucial test. The results were unequivocal: when $CaCl_2$ of the range 0.013-0.025 M was added to either 0.000001 M $CuCl_2$ + 0.000001 M Na_2SO_4 or 0.000002 M $CuSO_4$ + 0.000002 M NaCl, no neutralization of toxicity due to the copper salts was obtained. This is evidence that the sulfate radical (SO_4^{--}) so introduced has destroyed the power or capacity of $CaCl_2$ to neutralize the toxic action of the otherwise neutralizable $CuCl_2$.

Discussion. This work has demonstrated the toxicity of several metallic salts to *Paramecium*. It is reasonable to assume that others not employed in our experiments may be similarly toxic. Since a comparable pattern of toxicity is revealed by the protozoan under their action as well as that of our distilled water, a general mechanism may be involved in causing death of the organism. Our observations indicate that the ions cause an osmotic change, thereby injuring the plasma membrane in every case. On the other hand, specificity of the different ions is clearly shown by the fact that the different salts required different concentrations to duplicate the toxicity level of the distilled water.

The results have revealed 2 most significant observations: (a) that calcium chloride can neutralize the toxicity of distilled water, cupric chloride and lead acetate, but not the other 3 salts tested, and (b) that SO_4^{--} ions, if present together with calcium, destroys the latter's capacity to neutralize. These findings jointly led us to conclude that the unknown factor in question comprises possibly Cu^{++} and/or Pb^{++} and Cl^- ions. This conclusion is strengthened by the recent work of Bermes (3), who found that traces of the copper ion brought about oxidative changes in isolated human serum lipoproteins. Our results also substantiate previous reports on protective action of calcium against various deleterious effects to aquatic organisms, *e.g.*, marine fish, annelides, and flatworms(4,5,6,7,8).

There are indications that the anions in our experiments also exercise some toxicity to *Paramecium*. Assuming SO_4^{--} and Cl^- to be both mildly toxic, the fact that $CuCl_2$ is twice as toxic as $CuSO_4$ shows that Cl^- is the more toxic of the two. On this basis we may understand why complete neutralization of $CuCl_2$, for instance, was achieved by $CaCl_2$ at a concentration considerably below the latter's threshold toxic level, and that employment of amounts of calcium over this level invariably resulted in an enhanced instead of reduced toxicity. Evidently, in both these instances toxicity of the Cl^- ion plays a role.

Summary. A toxic factor present in our distilled water found lethal to *Paramecium* was analyzed. The results show that it is not related to pH, can be duplicated by 5 metallic salts at specific concentrations (M) as follows: cupric chloride, 10^{-6}; cupric sulfate and cupric nitrate, 20^{-6}; zinc acetate, 10^{-4}; lead acetate, 25^{-4}; and can be neutralized by 0.02 M $CaCl_2$. Calcium chloride is likewise capable of neutralizing cupric chloride and lead acetate, but not cupric sulfate, cupric nitrate or zinc acetate. These results coupled with the demonstration that the presence of SO_4^{--} ions actually destroys the neutralizing power of calcium indicate strongly that the toxic factor under consideration could possibly be copper chloride and/or lead chloride, not likely sulfate or nitrate of copper or zinc acetate. Generally the results also confirm the protective action of Ca^{++} against ionic imbalance or unknown impurities in water as reported by other workers. Because of the demonstrated sensitivity of *Paramecium* to metallic ions we commend its use as an indicator for low-level toxicity due to such ions in solutions including distilled water not made and stored in pyrex glass.

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