The addition of a chelating agent to water samples examined for pollution may be one answer to the problem of maintaining the coliform index near the level existing when a sample is taken. For periods up to 24 hours, a chelating agent materially reduced the "death rate" of coliform bacteria.

Chelation as a Method for Maintaining the Coliform Index in Water Samples

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THE POSSIBLE USE of a chemical chelating agent to preserve coliform bacteria in water samples examined for pollution has been explored in a series of experiments by the Tennessee Department of Public Health. Samples of various waters, inoculated with *Escherichia coli*, were tested to determine the rate of decrease in viable cells and the effect of a chelating agent on this rate of decrease.

The coliform bacteria are widely used as indicators of pollution in untreated waters, although at present this practice is a matter of some controversy. Evidence that coliform organisms multiply in waters containing organic matter has been reported by a number of workers, including Caldwell and Parr (*1*), Leahy (*2*), and Mallmann (*3*). However, there appears to be more evidence that the coliform index decreases rapidly during storage of samples, even during storage at low temperatures. Caldwell and Parr (*4*), Cox and Claiborne (*5*), the British Public Health Laboratory Service Water Subcommittee (*6*), and Leininger and McCleskey (*7*) have reported a material decrease of coliforms in samples after storage at various temperatures and for various periods of time.

These reports dealt primarily with temperature and time as factors influencing the decrease in coliforms. It seems reasonable, however, that the presence of certain chemicals in the water might also be a factor. The toxicity of polyvalent metallic ions for *E. coli* has been demonstrated by several workers, including Hotchkiss (*8*) and Fabian and Winslow (*9*). Waters receiving certain industrial wastes may well contain concentrations of metals sufficient to reduce the number of bacteria. A chelating agent added to samples taken from such waters would bind, or complex, any metallic ions present and would thereby prevent their deleterious effect on the bacteria.

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Materials

The chelating agent used in the experiments was Versene Regular, the tetrasodium salt of ethylenediamine tetra acetic acid (EDTA). This compound is one of several powerful amino acids and their salts which are useful as complexing agents for metal ions (*10*). These

acids have the ability to form soluble metal chelate compounds in which the polyvalent metal ion has been bound in a nonionic form. They are nonspecific; that is, they will inactivate practically any metallic ion with which they come in contact.

In a 1.0 percent solution, Versene Regular has a pH of 11.8. In order not to change materially the pH of the test waters, a 10^{-1} stock solution was prepared by combining 1 part Versene Regular with 8 parts double distilled water and 1 part 1N HCl. The pH of this solution was approximately 6.5. The neutralized EDTA 10^{-1} solution was sterilized by autoclaving for 15 minutes at 121° C.

The plating medium used was Bacto-tryptone glucose extract agar.

Escherichia coli ATCC 11229 was the inoculum used. All suspensions were prepared in buffered distilled water diluent (*11*) from cultures grown in nutrient broth for 24 hours at 37° C.

Effect of EDTA on E. coli

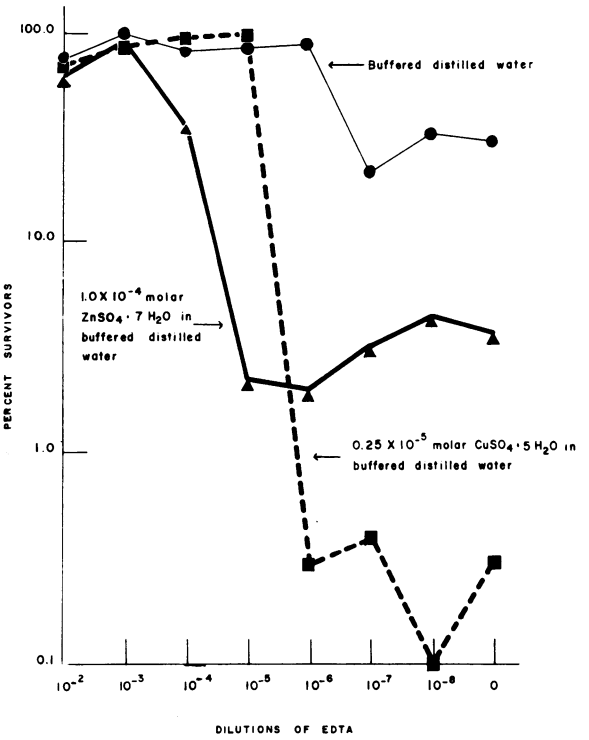
The first in the series of experiments was a study of the effect of the chelating agent on *E. coli*. For this study, varying log dilutions of EDTA in buffered distilled water were prepared. To each of these dilutions, tubed in 9.0-ml. aliquots, was added 1.0 ml. of a diluted cell suspension so that the test dilutions contained 200 to 300 cells per milliliter. These suspensions were kept at 25° C. for 2 hours and then plated to determine the viable cell count.

The light solid line in figure 1 shows the mean result of five trials. The 10^{-3} to 10^{-6} dilutions of EDTA gave a pronounced increase in recovery of cells over the recovery in cell suspensions containing no EDTA, that is, the buffered suspensions. This increase is indicative of an adverse effect of the buffer solution on *E. coli* under these conditions.

Chelation of Polyvalent Metals

The second in the series of experiments was a demonstration of the chelation of polyvalent metallic ions. Varying amounts of EDTA were combined with concentrations of metals known to be toxic to *E. coli* cells, and cells were then added to these chelated solutions.

Figure 1. Effect of EDTA on survival of Escherichia coli in several waters after 2 hours' exposure at 25° C.



Copper and zinc were the metals chosen for this demonstration. Stock solutions of 0.1 molar $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$ were prepared using double distilled water. These were further diluted with sterile buffered distilled water so that the final concentrations were 0.25×10^{-5} molar for the copper solution and 1.0×10^{-4} molar for the zinc solution, which correspond to concentrations of 0.16 and 6.5 p.p.m., respectively. These solutions were tubed in 8.0-ml. aliquots. Log dilutions of EDTA from 10^{-1} to 10^{-7} were prepared separately, and 1.0-ml. portions were added serially to the copper and zinc solutions. *E. coli* cell suspensions were added to each of the copper and zinc solutions containing varying amounts of EDTA so that the final cell concentration was approximately 200 to 300 cells per milliliter. These suspensions were held at 25° C. for 2 hours and then assayed for viable cell count.

The dotted line in figure 1 shows the mean result of five trials with the copper solution. The optimum range for chelation of the copper

solutions was 10^{-3} to 10^{-5} dilutions of EDTA. Above the 10^{-5} dilution there was a decided decline in recovery of viable cells because of insufficient EDTA and a resultant copper toxicity.

The mean result of five trials with the zinc solution is shown by the heavy solid line in figure 1. The optimum dilution of EDTA for chelation of zinc appears to be 10^{-3}. A gradual loss in cell recovery occurred as the dilution of EDTA increased.

Effect on Bacterial Death Rates

If fecal pollution enters water containing sufficient quantities of polyvalent metals to result in toxicity to the coliforms, samples collected from this water may well be free of coliforms by the time they reach the laboratory for analysis, even in a matter of a few hours. The addition of EDTA to such samples might interrupt this "death rate" at the time the sample is taken. To investigate this possibility, the following series of laboratory trials was made.

Cell suspensions of *E. coli* containing 2,000 to 3,000 cells per milliliter were prepared. Enough $CuSO_4 \cdot 5H_2O$ solution was added to these suspensions to produce a 0.25×10^{-5} molar (0.16 p.p.m.) solution. Immediately after addition of the $CuSO_4$, 10.0 ml. of the suspension was removed and placed in a tube containing 0.1 ml. of 10^{-1} dilution of EDTA. This tube was held at 25° C. for later analysis. After 10 minutes another 10 ml. was removed from the original suspension and added to a tube containing 0.1 ml. of the 10^{-1} dilution of EDTA. This was immediately assayed for viable cell count, and the remainder was held at 25° C. for subsequent analysis. Samples were taken at 20, 30, 60, and 120 minutes and treated as the sample taken at 10 minutes. All chelated samples were reassayed after 2 hours at 25° C. and again after 24 hours at the same temperature.

Similar trials were performed with a zinc solution, using 1.0×10^{-4} molar (6.5 p.p.m.) $ZnSO_4 \cdot 7H_2O$ as the final concentration of zinc.

Since buffered distilled water had shown an adverse effect over a 2-hour period, it was included in this study. Deionized (Crystalab Deeminizer) distilled water was also included. Samples of these waters were chelated at the various time intervals described.

Figure 2. Effect of EDTA on rate of decrease in Escherichia coli in 0.25×10^{-5} molar (0.16 p.p.m.) $CuSO_4 \cdot 5H_2O$ buffered distilled water.

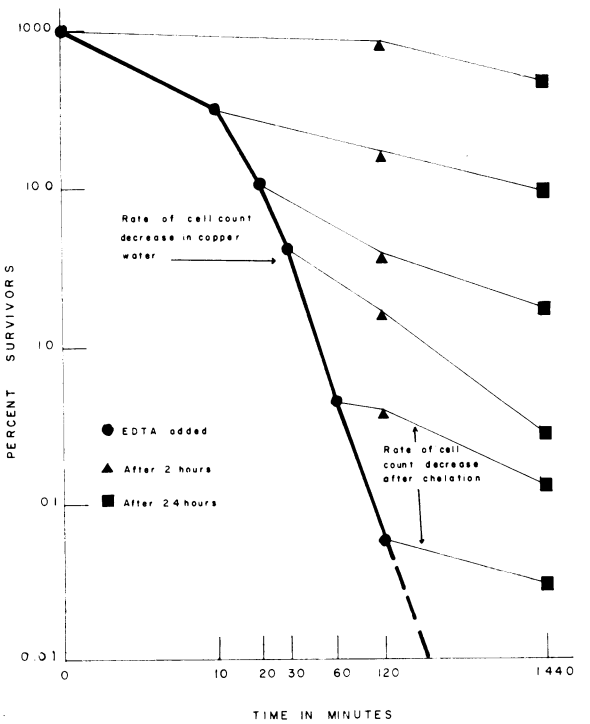
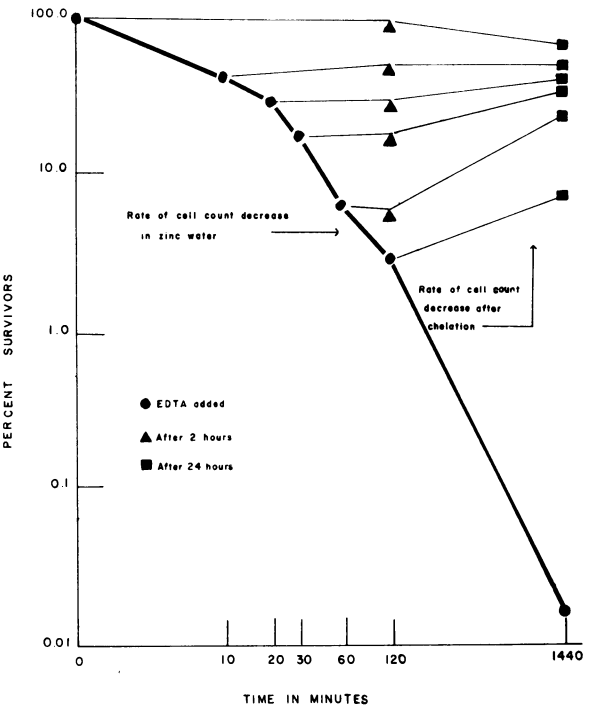


Figure 3. Effect of EDTA on rate of decrease in Escherichia coli in 1.0×10^{-4} molar (6.5 p.p.m.) $ZnSO_4 \cdot 7H_2O$ in buffered distilled water.



Ten trials were performed for each water tested.

In figure 2 the loss of viable cells exposed to copper is shown by the heavy line. The circles indicate the viable cell count at the time of chelation with EDTA; the triangles, the count in the chelated suspension after a period of 2 hours' exposure; and the squares, the count after 24 hours' exposure.

In an unchelated sample, the cells in the suspension apparently die off shortly after 2 hours' exposure. In the 2-hour samples, the rate of cell count decrease was definitely changed by the addition of EDTA. As exposure to copper continued, the slope of the line increased from time of chelation to the 2-hour period. This indicates that as exposure continues progressive damage may be done to the cells which cannot be overcome by chelation. The slope from 2 hours to 24 hours was about the same for all of the time intervals.

Figure 3 shows similar data for the zinc solution. Chelation of zinc apparently holds the count at approximately the same level as it is at the time of chelation. From the 24-hour samples, it can be surmised that some of the cells recover after standing in EDTA, or else the remaining cells are stimulated to multiply to a slight degree.

Figure 4. Effect of EDTA on rate of decrease in Escherichia coli in buffered distilled water.

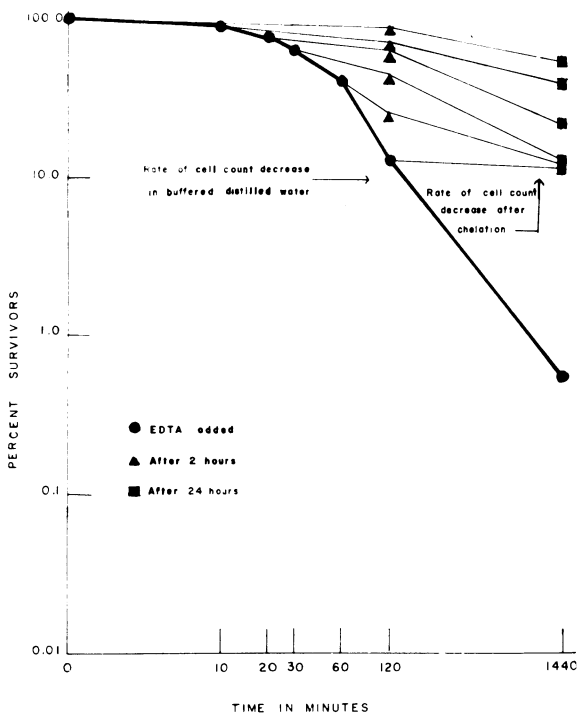
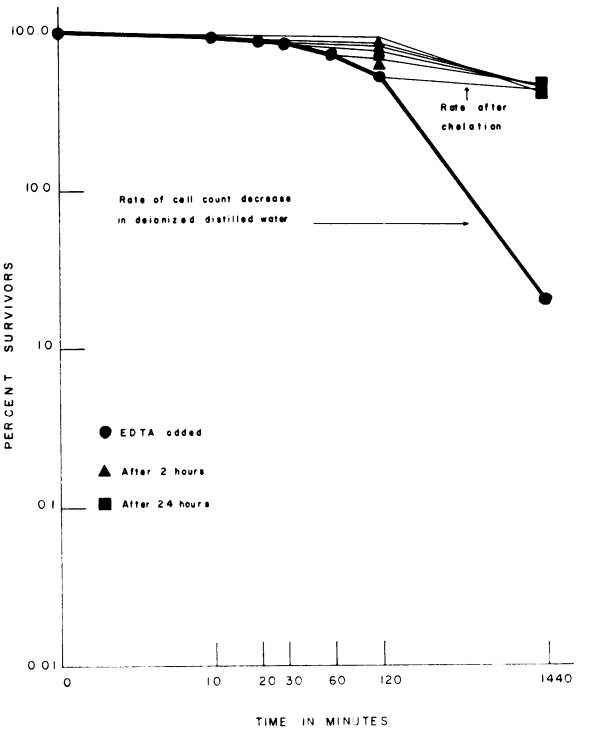


Figure 4 shows the results obtained when buffered distilled water was used as a suspending fluid. EDTA is apparently effective in reducing the rate of decrease in *E. coli* cells in buffered distilled water void of added metals.

Figure 5. Effect of EDTA on rate of decrease in Escherichia coli in deionized distilled water.



In figure 5 are shown the data for the deionized water. This material had the lowest "bacterial death rate" of any of the waters tested. Even so, the addition of EDTA to deionized water resulted in an improved recovery, possibly because of an osmotic pressure difference more favorable to the cells than deionized water.

The mean pH of the waters tested was 6.87 ± 0.13.

Discussion

These experiments have shown a rate of decrease in *E. coli* cells suspended in various waters. Waters containing small amounts of

metals, 0.16 p.p.m. of copper or 6.5 p.p.m. of zinc, were the most deleterious tested. Even buffered distilled water was responsible for loss of approximately 99.7 percent of the cells within a 24-hour period. In deionized water 98.0 percent of the cells were gone within 24 hours.

EDTA in dilutions of 10^{-2} to 10^{-6} was found to be nontoxic for *E. coli* cells. Greater dilutions were of no value in maintaining cell viability.

The Public Health Service Drinking Water Standard sets 3.0 mg. per liter (3.0 p.p.m.) as the upper limit for copper and 15.0 mg. per liter (15 p.p.m.) as the upper limit for zinc (*11*). Both of these limits exceed the amounts of these metals used in our experiments. Copper and zinc in the quantities used were shown to increase materially the rate of cell count decrease of *E. coli* as compared with the rate for buffered or deionized distilled water. Addition of EDTA to samples of these waters, taken at various time intervals, resulted in a reduced rate of decrease in the cell count, the amount of reduction depending upon the time the sample was taken. For example, in a sample from water containing copper (0.16 p.p.m.) the percentage of survivors was reduced to 0.08 after 2 hours and to approximately zero shortly thereafter. The addition of EDTA at the time of sampling resulted in approximately 69.0 percent of the cells remaining viable after 2 hours and 40.0 percent after 24 hours. Similar increased recovery was shown for the other waters tested.

Several trials, not described in this report, have been run on samples of rural water supplies. In some samples, the addition of EDTA seemed to promote growth of the coliforms when the samples were stored at 25° C., whereas without EDTA, there was a decrease in numbers of cells during storage. This effect of EDTA, of course, has its disadvantages, in that it tends to give an overestimation of coliform density. On the other hand, would it not be better to find positive those water sources that previously have been reported negative because of a loss of viable cells from the time the sample was taken until it was tested in the laboratory than to report them as safe supplies? The addition of EDTA to samples might be of value in the isolation of enteric pathogens from waters. Studies of this possibility are now being undertaken in this laboratory.

Summary and Conclusion

In laboratory experiments by the Tennessee Department of Public Health, the addition of the chelating agent ethylenediamine tetra acetic acid to samples of various waters (water containing copper or zinc, buffered distilled water, and deionized water) materially reduced the rate of decrease in *Escherichia coli* cells. It appears that, for periods up to 24 hours, chelating agents would be of value in maintaining the coliform index near the level existing at the time the sample is taken.

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