

EVALUATION OF CHEMICAL AND BACTERIOLOGICAL METHODS
OF DETERMINING GERMICIDAL ACTIVITY OF CHLORINE

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INTRODUCTION

Although chlorine has been used over half a century in disinfecting drinking water, swimming pool water, sewage and the surfaces of various objects, there is need of further research on the mechanism of action and methods of measuring germicidal activity of chlorine.

A number of methods of measuring chlorine residuals have been devised. The earliest method is the starch-iodide test which measures the chlorine residuals accurately when chlorine is introduced into chemically pure water. The ortho-tolidine method, which was developed later, is a colorimetric test giving equally good results when tests are made for total chlorine residuals. Both these tests measure all the active chlorine present, which includes free and combined chlorine. The latter test will give false results in the presence of interfering substances such as nitrites, etc.

The ortho-tolidine arsenite test eliminates the effect of interfering substances and also measures the free chlorine more accurately.

The ortho-tolidine test when used as a flash indicator measures free chlorine because the combined chlorine is released slowly.

In all these methods acidified solutions are used. The change in pH of the test solutions causes marked shifts in the chlorine equilibrium so that the chlorine residual obtained may be in excess of the active chlorine present in the original solution.

In the amperometric titration method sodium arsenite is used in neutral solution so there should be no disturbance of the chlorine equilibrium in the test solution. This test appears to be the best method of measuring free chlorine that has been devised to date.

The most accurate method of measuring active chlorine, however, would be a bacterial titration, based on the actual kill of bacteria introduced into the test solution. After all, the chlorine is introduced into water and sewage to render the product free of health hazard. This means that any measurement of residual chlorine should actually measure the bactericidal activity of the chlorine solution.

The ortho-tolidine arsenite and amperometric titration methods were used recently in the laboratory for measuring the germicidal chlorine present in swimming pool water in the Lansing area. It was observed, on a number of occasions, that high bacterial populations were present with relatively high chlorine residuals as measured by free chlorine, whereas low bacterial populations were ob-

tained with low chlorine, residuals although the bathing loads were comparable. This indicated the possibility that methods of testing were at fault.

Earlier it was demonstrated that the starch-iodide and ortho-tolidine tests failed to measure germicidal chlorine in the presence of suspended solids. Inasmuch as the swimming pool water was slightly turbid due to suspended solids, it appeared that both the ortho-tolidine arsenite and the amperometric tests might be measuring all or part of the chlorine which might be adsorbed on the suspended solids.

These studies were made to determine accordingly the accuracy of these methods, checking with bactericidal tests in the presence and absence of suspended solids of various types.

REVIEW OF LITERATURE

Because chlorine is used in minute quantities to destroy bacteria in water supplies, it is necessary that methods measuring germicidal residuals be very accurate. And further, because the chlorine is sometimes used in the presence of organic matter (soluble and suspended), salts, alkalies, and acids, it is necessary that the tests faithfully register germicidal chlorine only.

Originally, the starch-iodide titration method was applied. It was not until 1909 that a new method was suggested. According to Adams and Buswell (1), at that time an ortho-tolidine test was proposed by Phelps. This test was developed for practical use by Ellms and Hauser (5) in 1913. They showed the advantages of this test over those of the starch-iodide titration. They reported that the ortho-tolidine test as devised by them would detect as little as 0.005 ppm. available chlorine.

Adams and Buswell (1) reported that the ortho-tolidine frequently reacted slowly with the chlorine so that long contact periods between the chlorinated water and the orthotolidine reagent were necessary to measure the total chlorine residual. They recommended an exposure period of, at least, ten minutes.

Scott (24) showed that nitrites and ferric iron caused an interference in the ortho-tolidine test by producing false chlorine residuals. Monfort (19) reported that 7 ppm. ferric iron would produce color with ortho-tolidine. Scott (25) also reported that ferric iron in acid solutions produced a strong color with the starch-iodide test.

Tarvin, Todd and Buswell (29) reported that the ortho-tolidine test was affected by: the oxidation-reduction potential of the chlorine and chloramine, pH, reaction time and the concentration of chlorine, nitrites, hydrochloric acid and the reagent.

According to Griffin (7) the chief substances which interfere with the ortho-tolidine test are manganese, nitrites, iron and organic coloring matter. To the above interfering substances, Johnston and Edmonds (9) added ozone, nascent oxygen, ammonium persulphate, ferric chloride, ferric alum, potassium permanganate, potassium dichromate, sodium peroxide, bromine, iodine, and nitric acid. They also reported that algae interfere with the ortho-tolidine and starch-iodide tests.

Gilcreas and Hallinan (6) stated: "the interference by these substances generally imposes on the color resulting from the reaction with chlorine a similar color that

cannot be differentiated and falsely high chlorine content is therefore, indicated".

Beard and Kendall (3) and Tilley (30) both observed that organic matter interfered with the germicidal activity of chlorine.

Mallmann and Edwards (14) noted marked discrepancies in the activity of chlorine in unfiltered waters on bacterial populations. They ran laboratory tests in the presence of suspended organic and inorganic substances and found that both starch-iodide and ortho-tolidine tests gave chlorine residual far in excess of the actual germicidal chlorine present. The actual germicidal chlorine was determined by the kill of test organisms. They reported three types of chlorine which would be present under such conditions, namely, combined, adsorbed and free chlorine. Only the latter would be germicidal. Both starch-iodide and ortho-tolidine measured the adsorbed chlorine in addition to the germicidal chlorine.

It was apparent that neither the ortho-tolidine nor the starch-iodide test measures the actual germicidal or free chlorine. New tests or adaptation of the ortho-tolidine test were necessary. A number of new tests have been reported in the literature. Most of them appeared to have little value or they were

not an improvement over the ortho-tolidine.

Rideal and Evans (22) in 1921 suggested the oxidation-reduction potential concept of chlorination. Schmelkes (23) pointed out the relationship of the oxidation-reduction potential to the germicidal activity of chlorine.

In 1940 Mallmann and Ardrey (13) compared the oxidation-reduction potential, the ortho-tolidine test, the starch-iodide test and the bacterial kill titration as measurements of germicidal chlorine. They concluded that the oxidation-reduction potential gave a true picture of the germicidal activity of chlorine in the presence and absence of suspended organic matter. This study was an attempt to find a better method than the starch-iodide or ortho-tolidine tests for measuring chlorine residuals. No attempt was made by them to develop a test for general use. They were merely interested in finding whether an oxidation-reduction potential test would actually measure germicidal chlorine.

In 1944, Hallinan (8) and Gilcreas and Hallinan (6) introduced the ortho-tolidine arsenite (O.T.A.) test, which they reported, measures the active residual chlorine and chloramine in water. They found that by the use of this test they were able to eliminate interference from manganese or other interfering substances. Connel (4)

confirmed the finding of Gilcreas and Hallinan (6). The method was accepted by the Committee on Standard Methods for the Analysis of Water and Sewage, and appeared for the first time in the ninth Edition of Standard Methods (28).

Marks and Glass (17) made improvements on the amperometric titration method which was first reported by Kolt-hoff and Pan (11) for the titration of lead with dichromate and chromate. They used electrodes especially designed for the determination of chlorine in water. The method was compared with the ortho-tolidine test of Ellms and Hauser (5) and Muer and Hale (20). They reported that in the absence of iodide, free chlorine was determined. In the presence of potassium iodide both the free and the combined chlorine was obtained. They found that the titration was more accurate within pH limits of 6.5 to 8.0 and temperature limits of 32 to 77° F.

Baylis, Gerstein and Damann (2) reported that in 1942 the Wallace and Tiernan Company loaned a residual chlorine recorder to the Chicago Water Purification Division. The instrument was based on an electrometric principle involving the depolarizing action of chlorine on a copper electrode. They found the instrument convenient to use and adaptable to a variety of needs.

In 1947 Wallace and Tiernan Company (31) introduced

a new amperometric titrator which was used in the studies presented in this thesis.

Marks, Joiner and Strandskov (18) developed a new method for the use of the amperometric titrator for measuring chlorine in sewage effluents. The method used for measuring chlorine in water by the titrator is not applicable to sewage due to the fact that the iodine reacts too rapidly with mild reducing agents present in the sewage. They compared the ortho-tolidine method, with their modification of the amperometric titrator. Bacterial counts were made of the chlorinated effluents for comparative purposes.

EXPERIMENTAL

A. EXPERIMENTAL PROCEDURE

1. Preparation of zero chlorine-demand water.

The distilled water supplied to the laboratory contains a slight amount of ammonia. In order to obtain a zero chlorine-demand water, the distilled water was redistilled in a closed system; however, it was found that this water took up ammonia from the air even though care was exercised in the storage of the water. As soon as the glass stoppered bottles were opened the water showed a slight chlorine demand, and the demand gradually increased as the water was used. The use of double distilled water was found impractical for the intended uses.

Inasmuch as all distilled water available contained some ammonia, it was decided to prepare a zero chlorine-demand water by satisfying the chlorine demand of the laboratory distilled water.

The chlorine demand of the distilled water was determined by introducing graduated doses of chlorine in 0.1 ppm amounts into 500 ml. of distilled water and then measuring the chlorine residuals after 30 minutes contact.

Ten liters of distilled water was placed in a glass

stoppered bottle. A chlorine solution was added in sufficient amount to satisfy the chlorine demand of the water. After standing overnight the water was then checked by the starch-iodide, ortho-tolidine, ortho-tolidine arsenite and amperometric methods. The water gave a negative chlorine test. Tests were made for ammonia with Nessler's reagent (26), and a bacteriological test was made to demonstrate that the water had no germicidal activity.

2. Preparation of the chlorine stock solution.

The chlorine stock solution was prepared in a glass-stoppered amber bottle. Chlorine gas was bubbled into the water through plastic tubing. The stock solution was kept at 10° C. in a refrigerator. The strength of the solution was tested before each experiment. This solution contained from 550 to 650 ppm chlorine.

3. Starch-iodide titration method.

The reagents were prepared according to Standard Methods (28). Since the chlorine concentrations which were used varied between 1 and 10 ppm, 500 ml. samples were titrated. When, concentrations higher than 10 ppm (stock solution, and solutions for chemical controls) were titrated, dilutions were made accordingly.

For the iodometric titration 500 ml. of the sample

was placed in an Erlenmeyer flask and 10 ml. of 0.72N sulfuric acid solution was added. Then, 10 ml. of 7.5 percent potassium iodide solution was added and immediately mixed by a rotary movement and titrated with 0.01 N thio-sulfate solution. Toward the end of the titration, when the color of the sample being titrated became a faded yellow, 2.5 ml. of starch solution were added. This gave a deep blue color. After the addition of starch solution the titration was continued rapidly to a colorless solution. The amount of chlorine was then, calculated by the following equation:

$$\text{ppm. chlorine} = \text{ml. } 0.01 \text{ N Na}_2\text{S}_2\text{O}_3 \times 0.3546 \times \frac{1000}{500}$$

4. Ortho-tolidine flash method (27, 28).

Since the ortho-tolidine test is more accurate in concentrations of 0.1 to 1.0 ppm available chlorine all tests were made in these ranges. Where concentrations were above 1.0 ppm dilutions were made accordingly. A Wallace and Tiernan comparator was used. Fifteen ml. of the solution to be tested was placed in one cell for a control, and 14.25 ml. in the other. To this cell was added 0.75 ml. of ortho toolidine reagent. An immediate reading was made for free chlorine content. After five minutes contact a second reading was made and recorded as the combined-available chlorine.

5. Ortho-tolidine arsenite method (27, 28).

The advantage of this method over the ortho-tolidine test is the elimination of color produced by interfering substances. However, it has a disadvantage of giving high free available-chlorine residuals with samples containing a high proportion of combined chlorine, and, conversely, low free available chlorine with samples containing a low proportion of combined chlorine. These disadvantages are partially eliminated by keeping temperature and time constant between additions of reagents.

Three 15 ml. comparator cells were labelled A, B and OT. To cell A, 0.75 ml. of ortho-tolidine reagent and 13.5 ml. of the sample being tested were added and mixed with 0.75 ml. of arsenite reagent (27). A reading was made immediately for the free available chlorine (A value).

To cell B, 0.75 ml. of arsenite reagent, 13.50 ml. of sample and 0.75 ml. ortho-tolidine reagent were added respectively, mixed and the results recorded immediately (B-1 value).

A second reading was made in exactly five minutes (B-2 value). B-1 and B-2 represent the interfering colors in the immediate and five-minutes readings respectively.

The OT value was obtained in exactly the same manner

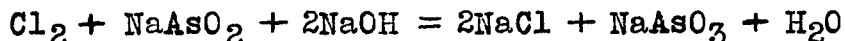
as that used for combined-chlorine in the ortho-tolidine test. The OT value represents the total amount of residual chlorine and the interfering substances present.

The total residual chlorine was obtained by subtracting the B-2 value from the OT value. The free available chlorine was obtained by subtracting the B-1 value from the A value.

The difference between total available chlorine and free-available chlorine gives the combined-available chlorine.

6. Amperometric titration method (31).

The principle of the operation of the amperometric titration of free chlorine, bromine, or iodine with sodium arsenite in neutral solution is based on a single quantitative reaction. This reaction for free chlorine is as follows:



The instrument contains a chlorine residual detection cell (11). The cell unit has a silver-positive electrode (anode) and a platinum-negative electrode (cathode). The anode is immersed in a saturated solution of sodium chloride which serves as a high conductivity bridge between the two electrodes. The cell is not affected by iron, manganese and nitrites because of the pH of the solution.

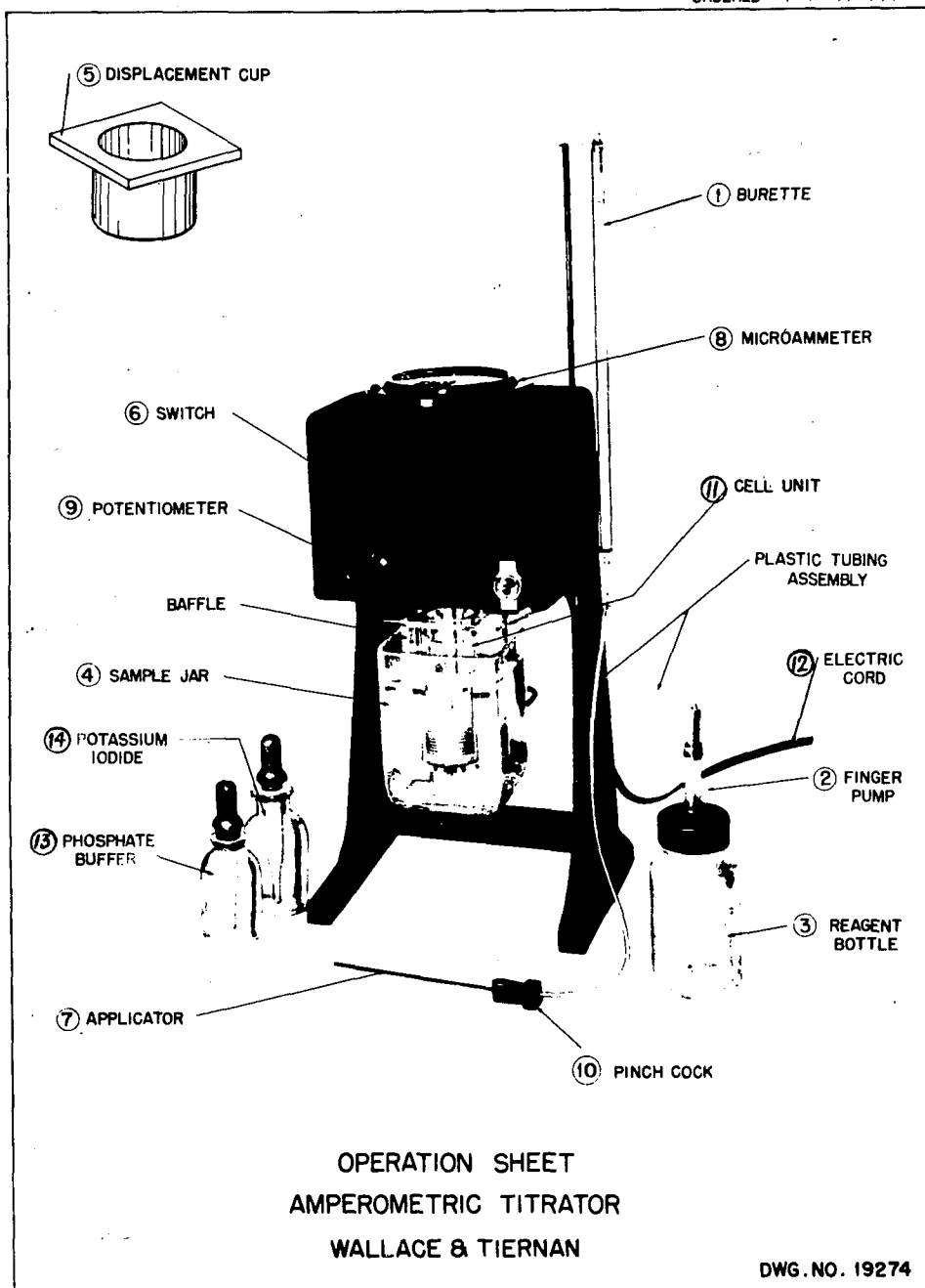
And since the cell has a relatively low internal resistance, dissolved salts, and chlorides have no effect on the cell.

The agitator helps to provide thorough and quick mixing of the sample being titrated with the sodium arsenite solution, and a continuous contact of the sample with the cathode.

The titration is carried out with 115 volt, single phase, 60 cycle current.

For the operation of the titrator, see accompanying photograph.

- a. The electric cord (12) was connected to an outlet.
- b. The burette (1) containing sodium arsenite was raised to its maximum height.
- c. Then, the burette was filled with the arsenite solution by pumping the finger pump (2).
- d. The sample jar (4) was filled with the solution being tested, and was brought up to the required amount by placing the displacement cup (5) on the top of the jar (200 ml. solution).
- e. The switch (6) was turned on to start the agitator.
- f. The pH was adjusted by adding one pipette full of phosphate buffer solution (13).
- g. Through the microammeter (8) the current was set at its maximum value by adjusting the potentiometer (9).



h. Then, the arsenite solution was added to the sample with the applicator (7) by squeezing the pinch cock (10). All through the titration the applicator was kept a few millimeters below the surface of the solution in the sample jar (4). Between the additions of sodium arsenite there was a pause so that the microammeter needle stopped moving.

When very large amounts of residual chlorine were titrated, a measured amount of arsenite solution was added with a pipette, then, the addition of sodium arsenite was continued, using the applicator.

A current was produced as long as a free chlorine residual was present. The current was in direct proportion to the free chlorine.

Addition of sodium arsenite, reduced the amount of free chlorine present in the solution, and this caused a decrease in current. When, all the free chlorine was combined with the sodium of the sodium arsenite, further addition of the sodium arsenite did not cause any change in current.

The end-point of the titration was indicated by the microammeter's (8) needle.

The total amount of sodium arsenite which was re-

quired to reach the end-point, gave the free chlorine which was present in the sample titrated. Each 0.1 ml. of sodium arsenite used indicates 0.1 ppm. free available chlorine.

- i. After all the free chlorine was titrated, titration of combined residual chlorine was accomplished. One drop of 5% solution of potassium iodide (14) was added. Potassium combined with chlorine and iodine was released. This caused the ammeter needle to swing all the way to the left or to fluctuate. Within a few minutes the needle returned to its normal position. Then, the titration preceeded as described for free-available chlorine. And the amount of the combined-available chlorine was read from the burette.

7. Preparation of buffer solution for bacteriological test (12).

A phosphate buffer solution containing sodium thio-sulfate was used for the neutralization of chlorine in the bacteriological test. The phosphate buffer of pH 7.0 was prepared as follows: Mono potassium phosphate (9.08 grams) was dissolved in distilled water and diluted to one liter. Disodium phosphate (9.47 grams) was added to distilled water and diluted to one liter. The final buffer was prepared by mixing 389 ml. of the monopotassium

phosphate solution with 611 ml. of disodium phosphate solution.

The pH of the solution was checked with a Beckman pH meter.

The dilution tubes were prepared by using 9 ml. of the buffer solution and 1 ml. of 0.1 N sodium thiosulfate. The tubes were sterilized in an autoclave at 120° C. for 20 minutes.

8. Bacteriological method (15).

The bacteriological tests were made by introducing into flasks 500 ml. of zero chlorine-demand water, the various foreign substances, dextrose, agar-agar, gelatin, diatomaceous earth, activated charcoal or skim milk. The flasks were then sterilized by autoclaving and placed in a water bath held at $25.0 \pm 1.0^{\circ}$ C. When the flasks had reached the temperature of the bath a known amount of chlorine solution was introduced into each flask. Flasks without foreign substances were used as controls. After 24 hours 1 ml. of Escherichia coli or Micrococcus pyogenes var. aureus suspension was added to each flask. This amount gave approximately 100,000 cells per ml. of solution. The suspensions of bacteria were prepared by adding the growth from a 24 hour tryptone-glucose extract agar slant to sterile water. The stock

suspension was standardized with the nephelometer (10) so that the suspension contained approximately 300,000,000 bacteria per ml. This was diluted so that the final stock solution contained approximately 50,000,000 bacteria per ml.

The bacteria were exposed to the action of the chlorine for periods of 15, 30, 45, 60, 90, 120 and 180 seconds. At the end of each interval one ml. was removed from the flask and introduced into 9 ml. of the buffer-thiosulfate solution to stop the action of the disinfectant.

Appropriate dilutions were made in sterile distilled water, and plated using T.G.E. agar. The plates were incubated at 25° C. for 24 hours and the number of surviving bacteria were determined.

Control tests were made by checking flasks containing the foreign substances and no chlorine to determine the original number of organisms introduced into the flasks. A control was made using sterile distilled, zero chlorine-demand water.

Simultaneously, to determine the germicidal activity of free chlorine in dosages of 0.1 to 1.0 ppm. in steps of 0.1 ppm., tests were made in zero chlorine-demand distilled water with the same number of organisms at the same

exposure intervals. The latter data supplied a set of death curves which could be compared with those obtained from the chlorine flasks containing foreign substances.

B. EXPERIMENTAL DATA AND DISCUSSION

The literature dealing with the relationship of the starch-iodide, ortho-tolidine flash, ortho-tolidine arsenite and amperometric titration methods for the measurement of chlorine is largely a comparison of one method with another. When attempts were made to correlate the results of these tests with germicidal activity of chlorine, the tests were based on the absence or presence of indicator organisms in a treated water supply. No bacteriological titrations were used.

These methods have been used for titrating chlorine in the presence of organic and inorganic (soluble or suspended) substances, but no accurate bacteriological titrations were employed as controls.

Mallmann and Edwards (14) studied the influence of suspended solids on germicidal chlorine as measured by the starch-iodide and ortho-tolidine tests. The germicidal activity of the chlorine was measured by bacteriological titrations using Esch. coli. They found that the actual germicidal chlorine as measured by bacterial kill was much lower than that indicated by either of the chemical methods used. They showed that the chemical methods were faulty in the presence of suspended solids, such as charcoal and

agar-agar because the adsorbed chlorine was apparently not germicidal as long as it remained adsorbed to the suspended solids.

The starch-iodide and ortho-tolidine flash tests measure the adsorbed chlorine in addition to the free chlorine, because the acid introduced causes a marked shift in the acidity of the test solution causing the adsorbed chlorine to be dispersed from the suspended particles. If the chlorine equilibrium had not been disturbed so that the chlorine adsorbed to the suspended solids remained attached, then the only chlorine capable of killing the bacteria would be the free chlorine.

No bacterial tests were made for chlorine similar to those made by Mallmann and Edwards (14) when the ortho-tolidine arsenite or the amperometric tests were checked. Inasmuch as, Mallmann and Seligmann (16) have observed marked discrepancies in chlorine residuals and bacterial kills in swimming pools when chlorine residuals were measured by the ortho-tolidine arsenite and amperometric methods, they suspected that chlorine adsorbed on suspended particles might be responsible.

The studies in this thesis follow somewhat the methods used by Mallmann and Edwards (14) except that the ortho-tolidine arsenite and amperometric procedures were

included. Also additional refinements were made to be sure that only free chlorine was present when obtaining the bacterial kill curves for measuring chlorine concentrations. M. pyogenes var. aureus was included, in addition to Esch. coli, so that both gram positive and gram negative bacteria were used with the various germicidal concentrations of chlorine.

1. The Bacteriological Method of Determining Germicidal Activity of Chlorine in Zero Chlorine-Demand Water.

In the first series of tests bacterial death curves for various chlorine residuals ranging from 0.1 to 1.0 ppm. by 0.1 stages were made, using both Esch. coli and M. pyogenes var. aureus. The bacterial kill was determined for exposures of 15, 30, 45, 60, 90, 120 and 180 seconds. The initial seeding of bacteria was approximately 100,000 per milliliter. The tests were made in sterile distilled, zero chlorine-demand water.

Care was exercised to be sure that each test was made under exactly the same conditions so that the only variable in each flask was the concentration of the chlorine. Eleven flasks containing 500 ml. sterile distilled, zero chlorine-demand water were held in a water bath at $25.0 \pm 1.0^{\circ}$ C. To flask No. 1 was added sufficient chlorine solution to produce 0.1 ppm. of available chlorine as deter-

mined by previous checking by chemical titrations. Each succeeding flask received an added increment of chlorine solution to yield a residual of 0.1 ppm. higher than the previous flask. A duplicate set of flasks was prepared for chemical titrations by the various methods under study. To the first set of flasks was added the appropriate test organism and death rates were determined by the previously described procedures.

The tests were repeated until 18 trials had been made with Esch. coli and 20 trials with M. pyogenes var. aureus. Most of the tests were made in parallel with tests in the presence of interfering organic substances so that the control death curves would be comparable. Thus it would be possible to apply the death curves obtained for the known amount of chlorine to those obtained for the flask containing the organic substance and the unknown amount of germicidal chlorine.

In Tables I and II and Figures I and II, are presented the average (arithmetic) percentage survival of Esch. coli and M. pyogenes var. aureus in the presence of varying chlorine residuals. Except for the first four runs, the percentage-survival figures for each exposure period and chlorine residual were similar. Because the first four runs varied markedly from later ones, they were discarded.

TABLE I

The Average Percent Survival of Escherichia coli in Various Concentrations of Chlorine at pH 7.0 ± 0.1 at $25.0 \pm 1.0^{\circ}$ C.

Obtained from Eighteen Trials.

PPM. Cl.	Percent survival of bacteria							
	Exposure time in seconds							
	0	15	30	45	60	90	120	180
0.1	100	99.1	86.3	72.8	57.0	42.8	25.0	3.0
0.2	100	95.6	76.6	57.6	41.9	28.4	14.7	1.6
0.3	100	92.4	70.6	53.3	31.1	20.6	6.5	0.4
0.4	100	89.3	66.2	46.3	28.4	10.1	3.4	0
0.5	100	85.7	61.3	34.9	23.3	6.4	0.1	0
0.6	100	81.5	57.5	32.6	18.1	2.2	0	0
0.7	100	75.8	48.5	24.8	10.5	0.5	0	0
0.8	100	59.6	32.4	10.5	4.1	0	0	0
0.9	100	27.3	9.1	0	0	0	0	0
1.0	100	8.8	0.4	0	0	0	0	0

TABLE II

The Average Percent Survival of Micrococcus pyogenes var.
Aureus in Various Concentrations of Chlorine at pH 7.0 ± 0.1
 at $25.0 \pm 1.0^{\circ}$ C. Obtained from Twenty Trials.

PPM. Cl.	Percent survival of bacteria							
	Exposure time in seconds							
	0	15	30	45	60	90	120	180
0.1	100	95.0	86.1	77.3	68.2	53.0	40.8	24.3
0.2	100	91.6	83.3	71.9	59.6	41.2	27.4	14.6
0.3	100	88.1	79.9	66.5	55.7	34.1	21.5	5.0
0.4	100	84.9	73.7	59.2	43.3	24.7	13.1	1.0
0.5	100	82.3	71.3	53.3	39.3	21.1	6.2	0.1
0.6	100	78.4	64.5	46.5	33.6	15.8	3.4	0
0.7	100	75.4	62.3	39.0	22.7	9.0	2.2	0
0.8	100	65.9	45.0	18.4	10.1	1.7	0	0
0.9	100	51.3	21.5	11.2	2.3	0	0	0
1.0	100	14.2	6.1	2.0	0	0	0	0

FIGURE I

The average percent survival of *Escherichia coli* in various concentrations of chlorine at $\text{pH } 7.0 \pm 0.1$ and $25.0 \pm 1.0^\circ \text{C}$. obtained from eighteen trials

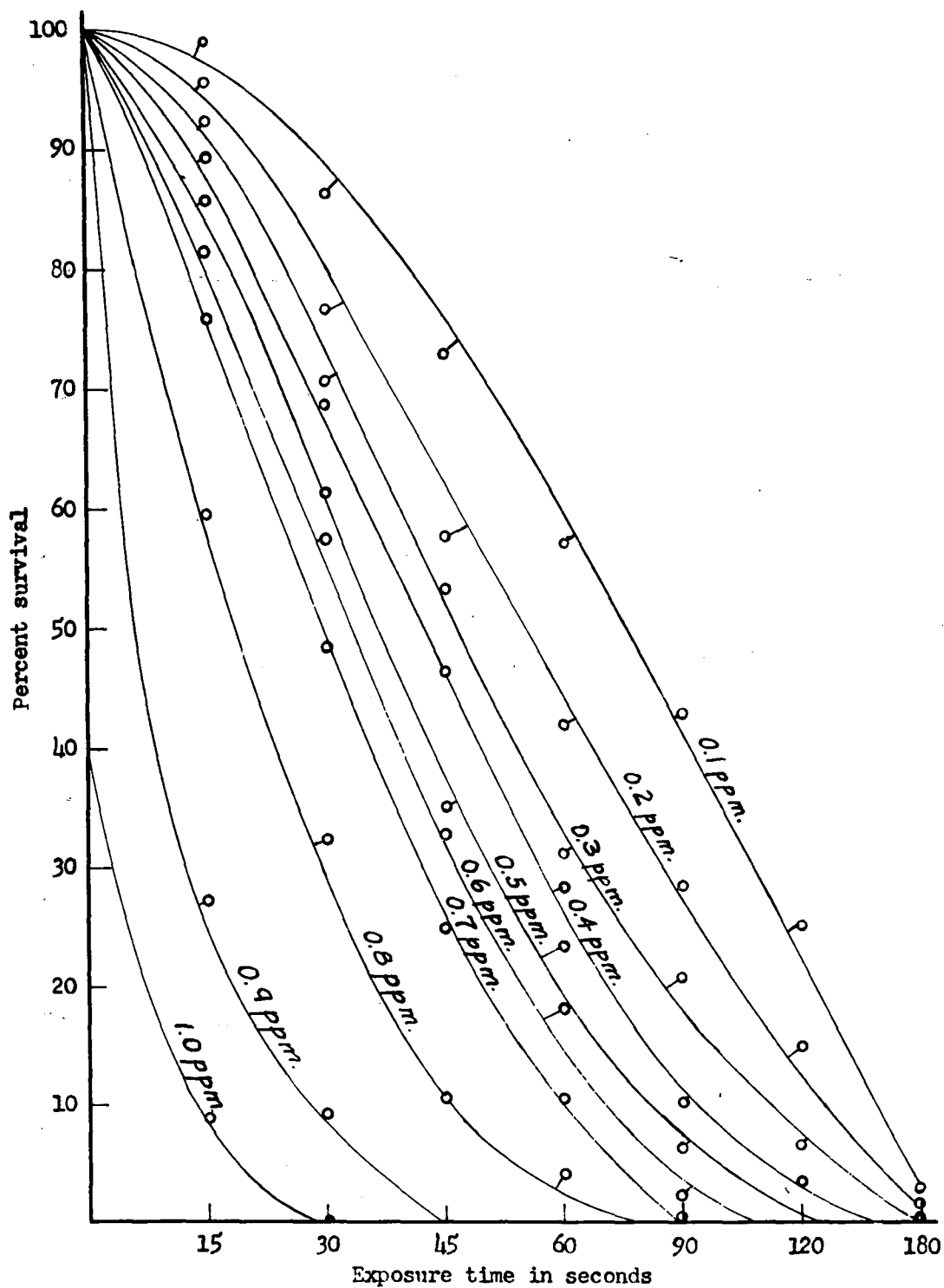
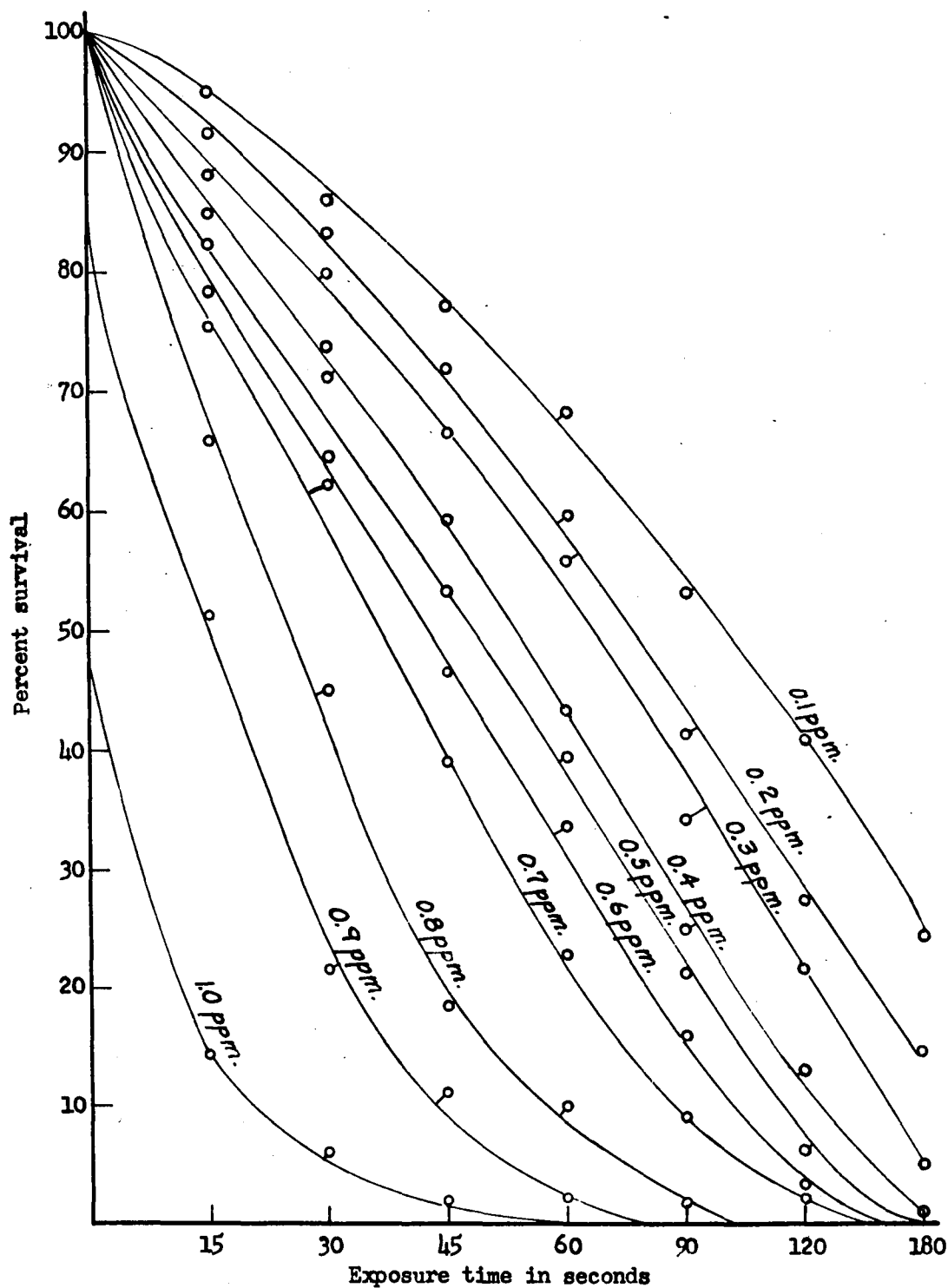


FIGURE II

The average percent survival of *Micrococcus pyogenes* var. *aureus* in various concentrations of chlorine at pH 7.0 ± 0.1 and $25.0 \pm 1.0^\circ$ C. obtained from twenty trials



The variability obtained in these early tests was likely due to inexperience.

At first it was planned to check the results of each series of tests in the presence of interfering substance with the control death curves prepared at the same time. However, because the control curves for each run were practically identical throughout the entire study, the averaged percentage-survivals were used for comparative purposes.

The fact that comparable death curves for each chlorine residual could be reproduced day after day indicates that a bacterial titration method can be used, at least, as a research procedure for the assay of germicidal chlorine in water solutions.

2. The Effect of Organic and Inorganic Matter in Chlorine Solutions upon Various Methods of Measuring Germicidal Chlorine.

Having demonstrated the dependability of the bacterial titration method for measuring germicidal chlorine tests were made in the presence of various interfering substances.

The first interfering substance used was dextrose, which was added to each flask so that the final concentra-

tion was 0.2 percent. Chlorine solution was added so that concentrations of 6.0, 10.0 and 15.0 ppm. were obtained. These flasks were then held in a water bath at $25.0 \pm 1.0^{\circ}$ C. for 24 hours. Two sets of flasks were prepared. The flasks were plugged with cotton stoppers. One set of flasks was checked for chlorine residuals using the various chemical procedures. The other set of flasks was adjusted to $\text{pH } 7.0 \pm 0.1$ and bacterial titrations were made. Because of the number of flasks involved in each run, it was necessary to make tests with Esch. coli on one day and tests with M. pyogenes var. aureus on the next. Each day, duplicate sets of flasks were prepared, one set for chemical titrations and the other for bacterial titrations.

The results are presented in Table III and Figures III and IV. Flask A showed 0.25 ppm. free chlorine by the ortho-tolidine flash test and 0.21 ppm. free chlorine by the amperometric method. The bacterial titration curve for dextrose solution follows closely that for 0.15 to 0.20 ppm. chlorine. Thus it would appear that a soluble substance, such as dextrose, does not interfere in the measurement of germicidal chlorine as determined by free chlorine tests.

The chemical titration also showed that the total chlorine tests of 3.80 ppm. for starch-iodide, 2.45 ppm. for ortho-tolidine flash and ortho-tolidine arsenite and

TABLE III

The Germicidal Activity of Various Concentrations of
Chlorine in the Presence of 0.2 Percent Dextrose at pH 7.0 $\pm$
0.1 and 25.0 $\pm$ 1.0° C.

Chemical Titration of Chlorine									
No. of Trls.	Flask No.	PPM. Cl.	I. test	O. T. test		O. T. A. test		Amperometric test	
			total	free	comb.	free	comb.	free	comb.
7	A	6	3.80	0.25	2.20	0.25	2.20	0.21	2.80
8	B	10	5.69	2.25	2.76	2.25	2.76	2.56	2.85
6	C	15	9.00	8.40	2.78	8.41	2.69	9.10	2.87

No. of Trls.	Flask No.	Bacteriological test							
		Exposure time in seconds							
		0	15	30	45	60	90	120	180

Percent survival of <i>Escherichia coli</i>									
4	A	100	96.8	81.5	65.0	45.6	37.1	25.1	10.2
4	B	100	9.1	0	0	0	0	0	0
3	C	100	0	0	0	0	0	0	0

Percent survival of <i>Micrococcus pyogenes</i> var. <i>aureus</i>									
3	A	100	96.4	88.4	78.6	72.2	57.3	40.8	21.2
4	B	100	8.5	2.6	0	0	0	0	0
3	C	100	0	0	0	0	0	0	0

Bacteriological controls on Tables I & II.

FIGURE III

The germicidal activity of various concentrations of chlorine in the presence of ± 0.2 percent dextrose at pH 7.0 ± 0.1 and $25.0 \pm 1.0^\circ \text{C}$.

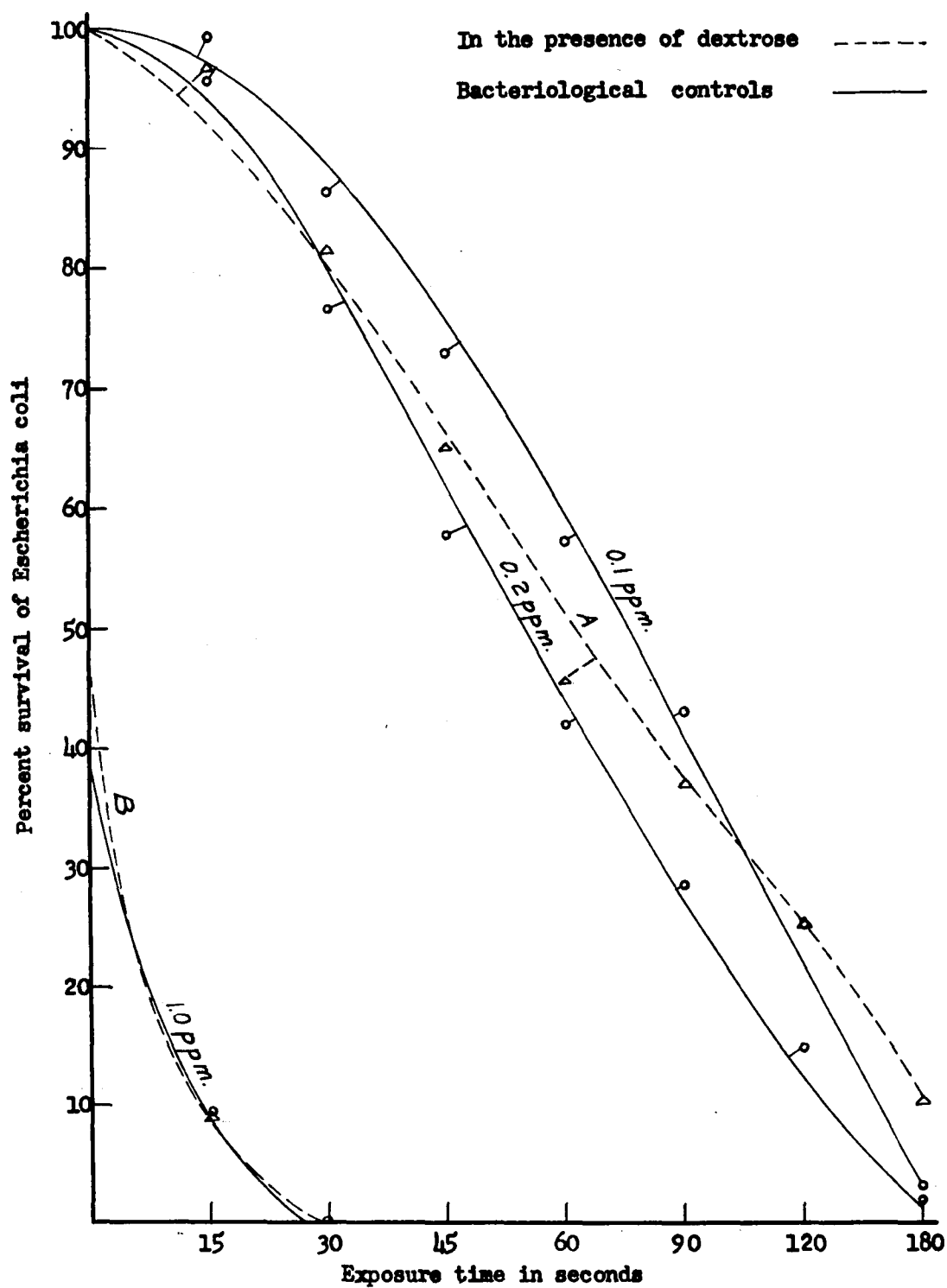
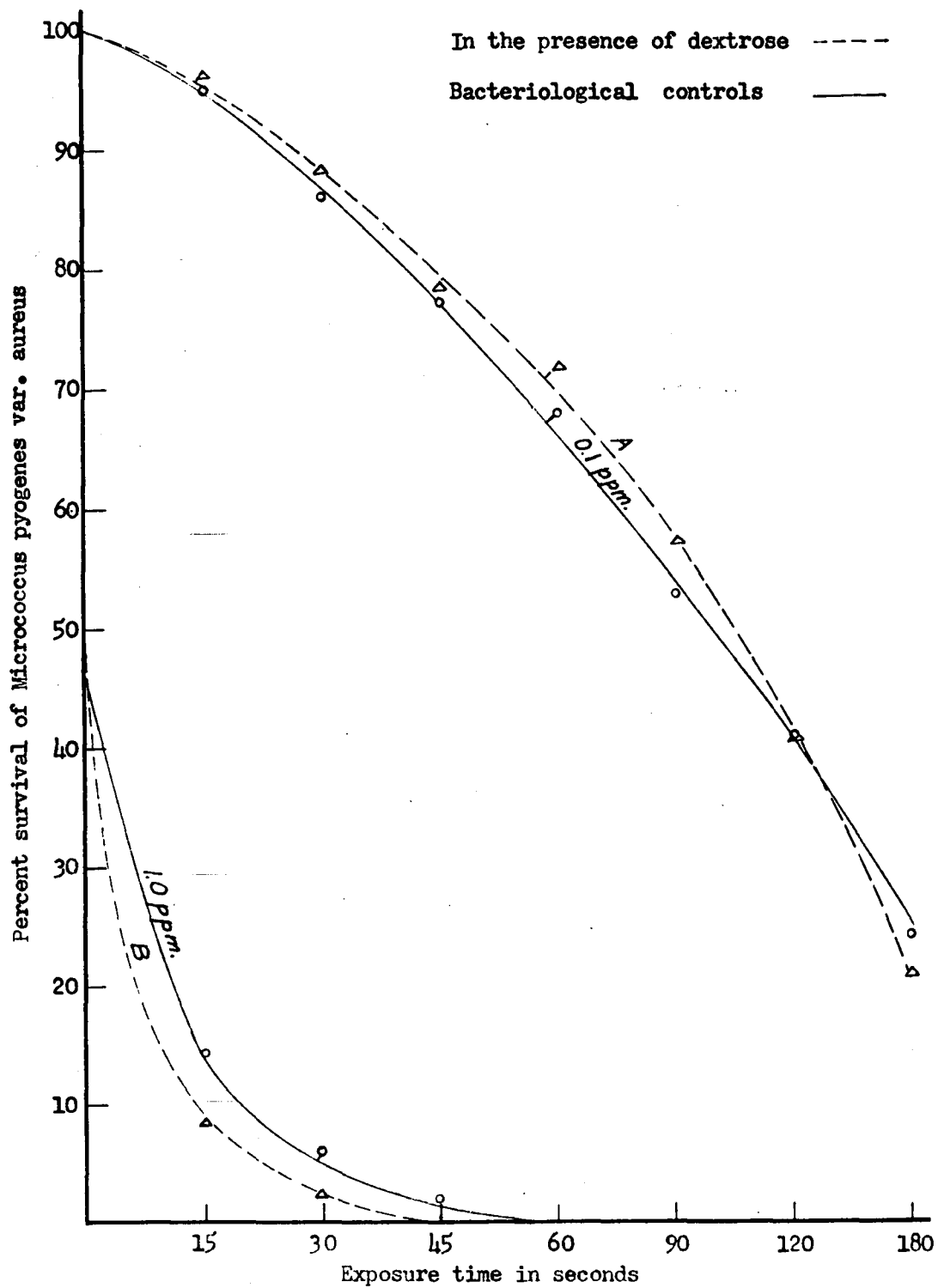


FIGURE IV

The germicidal activity of various concentrations of chlorine
in the presence of 0.2 percent dextrose at pH 7.0 ± 0.1 and
 $25.0 \pm 1.0^\circ \text{C}$.



3.01 ppm. for the amperometric titration were definitely not a measurement of germicidal chlorine.

These data are interesting because they confirm by bacterial titration the fact that the ortho-tolidine flash test and the amperometric titration gave dependable results under the conditions established in this experiment.

In the next series, a colloidal material was introduced. An ammonia-free agar-agar was added to the flasks in a final concentration of 0.1 percent. Chlorine was added to the flasks in concentrations of 6.0, 15.0 and 20.0 ppm. The experiments were conducted as in the preceding tests.

The data are presented in Table IV and Figures V and VI. Flask B, according to the bacterial titration, contained approximately 0.35 ppm. of germicidal chlorine. The free chlorine residuals as measured chemically were 1.22 ppm. for both the ortho-tolidine flash and the ortho-tolidine arsenite and 0.96 ppm. for the amperometric test. The tests for free chlorine were three to four times higher than the actual germicidal chlorine.

These data showed that the free chlorine test by any of the chemical methods used does not measure germicidal chlorine in the presence of a colloid, such as agar-agar.

TABLE IV

The Germicidal Activity of Various Concentrations of Chlorine
in the Presence of 0.1 Percent Agar-agar at pH 7.0 ± 0.1 and
 $25.0 \pm 1.0^\circ \text{C}$.

No. of Trls.	Flask No.	PPM. Cl.	Chemical titration of chlorine						
			S.I. test	O. T. test		O. T. A. test		Amperometric test	
			total	free	comb.	free	comb.	free	comb.
6	A	6	1.96	0.40	2.02	0.40	2.02	0.39	1.86
7	B	15	3.53	1.22	2.75	1.22	2.75	0.96	3.94
10	C	20	7.98	2.50	3.50	2.55	3.50	2.06	6.00

No. of Trls.	Flask No.	Bacteriological test							
		Exposure time in seconds							
		0	15	30	45	60	90	120	180

Percent survival of Escherichia coli									
3	A	100	97.6	88.2	78.3	68.0	58.3	50.9	48.8
3	B	100	84.5	77.0	54.2	36.3	15.6	4.6	1.1
5	C	100	66.6	49.7	22.4	11.5	2.4	0.5	0

Percent survival of Micrococcus pyogenes var. aureus									
3	A	100	98.1	92.4	85.5	76.7	72.0	61.7	59.8
4	B	100	86.3	67.9	54.5	41.2	33.6	20.2	12.5
5	C	100	61.7	38.9	20.8	10.4	3.1	0.7	0.04

Bacteriological controls on Tables I & II.

FIGURE V

The germicidal activity of various concentrations of chlorine in the presence of 0.1 percent agar-agar at pH 7.0 ± 0.1 and $25.0 \pm 1.0^\circ \text{C}$.

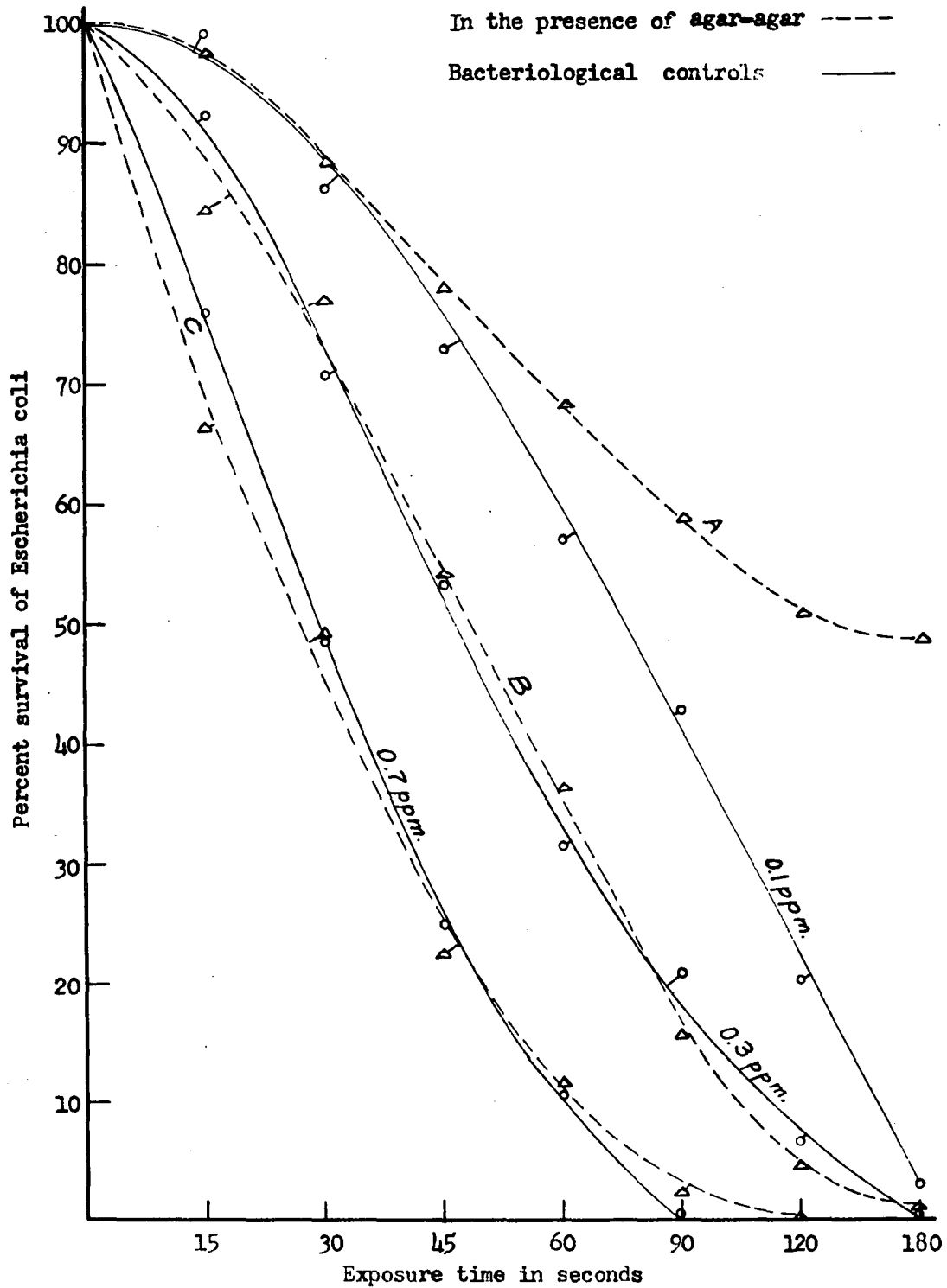
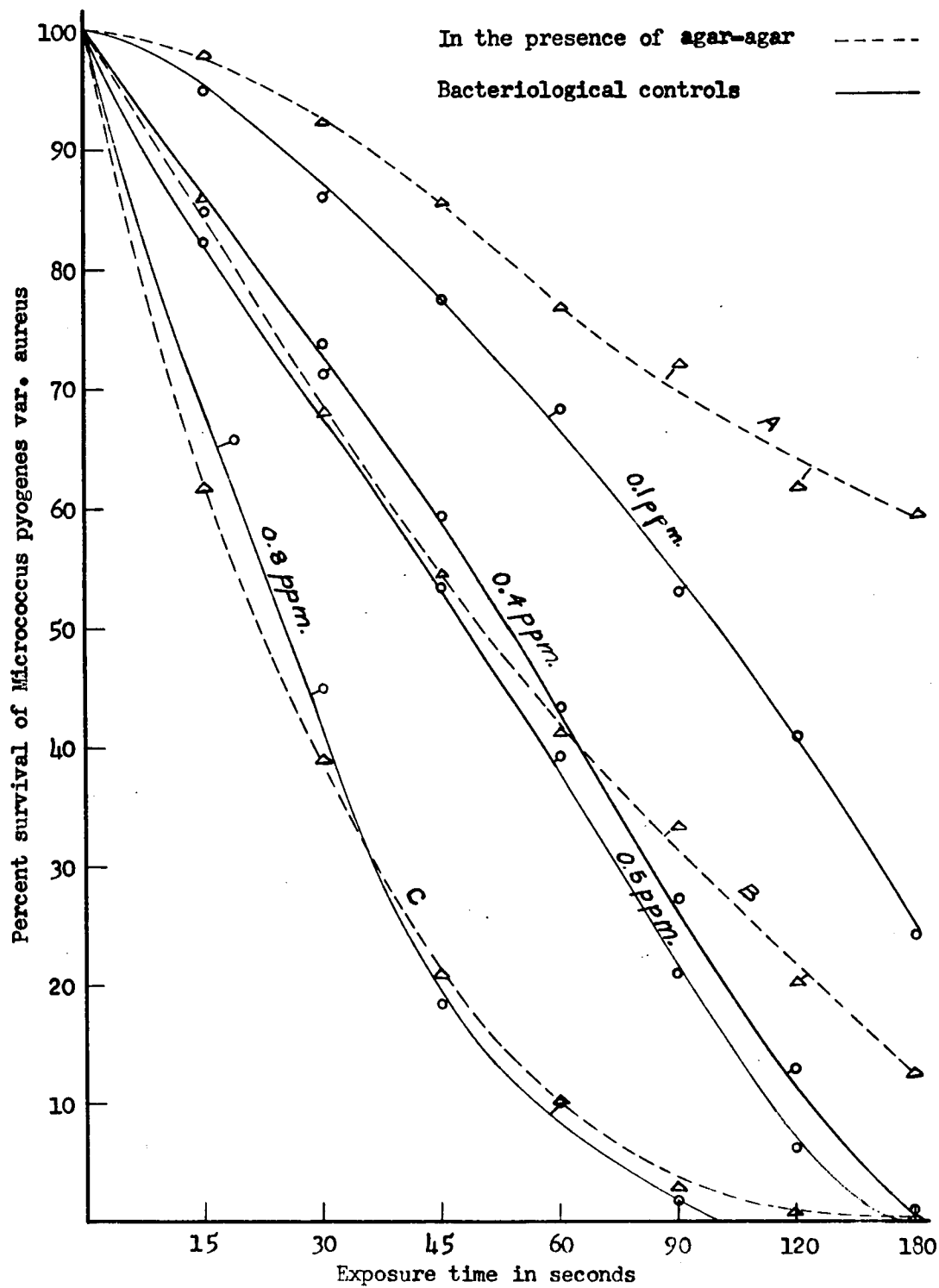


FIGURE VI

The germicidal activity of various concentrations of chlorine in the presence of 0.1 percent agar-agar at $\text{pH } 7.0 \pm 0.1$ and $25.0 \pm 1.0^\circ \text{C}$.



These data confirm the earlier findings of Mallmann and Edwards (14), who found that the starch-iodide and ortho-tolidine tests did not measure germicidal chlorine in the presence of agar-agar.

The above workers suggested that chlorine may take three forms in the presence of suspended materials, namely, chemically combined, adsorbed and free chlorine. They reported that the adsorbed chlorine was not germicidal but it was measured by both the starch-iodide and ortho-tolidine tests.

Gelatin was selected as another type of colloid. An ammonia-free gelatin was added to the flasks in a final concentration of 0.05 percent. Chlorine was then added in concentrations of 10.0, 15.0 and 20.0 ppm. The tests were made as before.

The data are presented in Table V and Figures VII and VIII. The bacterial titration of Flask B showed approximately 0.30 to 0.40 ppm. of germicidal chlorine. The chemical titration for free chlorine in Flask B was 0.80 ppm. for the ortho-tolidine flash and the ortho-tolidine arsenite tests and 0.00 ppm. for the amperometric method. Again the actual germicidal chlorine did not check with the free chlorine tests. The ortho-tolidine flash and the ortho-tolidine arsenite tests gave figures nearly three

TABLE V

The Germicidal Activity of Various Concentrations of Chlorine
in the Presence of 0.05 Percent Gelatin at pH 7.0 ± 0.1 and
 $25.0 \pm 1.0^\circ \text{C}$.

Chemical titration of chlorine									
No. of Trls.	Flask No.	PPM. Cl.	S.I. test	O. T. test		O. T. A. test		Amperometric test	
			total	free	comb.	free	comb.	free	comb.
7	A	10	2.77	0.56	2.52	0.58	2.51	0	3.25
9	B	15	4.88	0.80	3.76	0.80	3.77	0	4.50
8	C	20	9.11	1.06	7.64	1.08	7.64	0.05	8.35

No. of Trls.	Flask No.	Bacteriological test							
		Exposure time in seconds							
		0	15	30	45	60	90	120	180

Percent survival of <i>Escherichia coli</i>									
3	A	100	98.1	92.6	83.1	67.2	38.6	21.0	13.0
4	B	100	88.6	77.4	60.0	38.0	16.1	11.5	1.1
4	C	100	73.2	57.9	24.8	10.0	1.3	0	0

Percent survival of <i>Micrococcus pyogenes</i> var. <i>aureus</i>									
4	A	100	90.9	85.4	78.6	65.7	54.4	46.5	34.6
5	B	100	82.6	62.8	53.7	44.0	36.0	21.9	12.2
4	C	100	61.8	39.0	19.6	10.1	2.9	0.34	0

Bacteriological controls on Tables I & II.

FIGURE VII

The germicidal activity of various concentrations of chlorine in the presence of 0.05 percent gelatin at pH 7.0 ± 0.1 and $25.0 \pm 1.0^\circ \text{C}$.

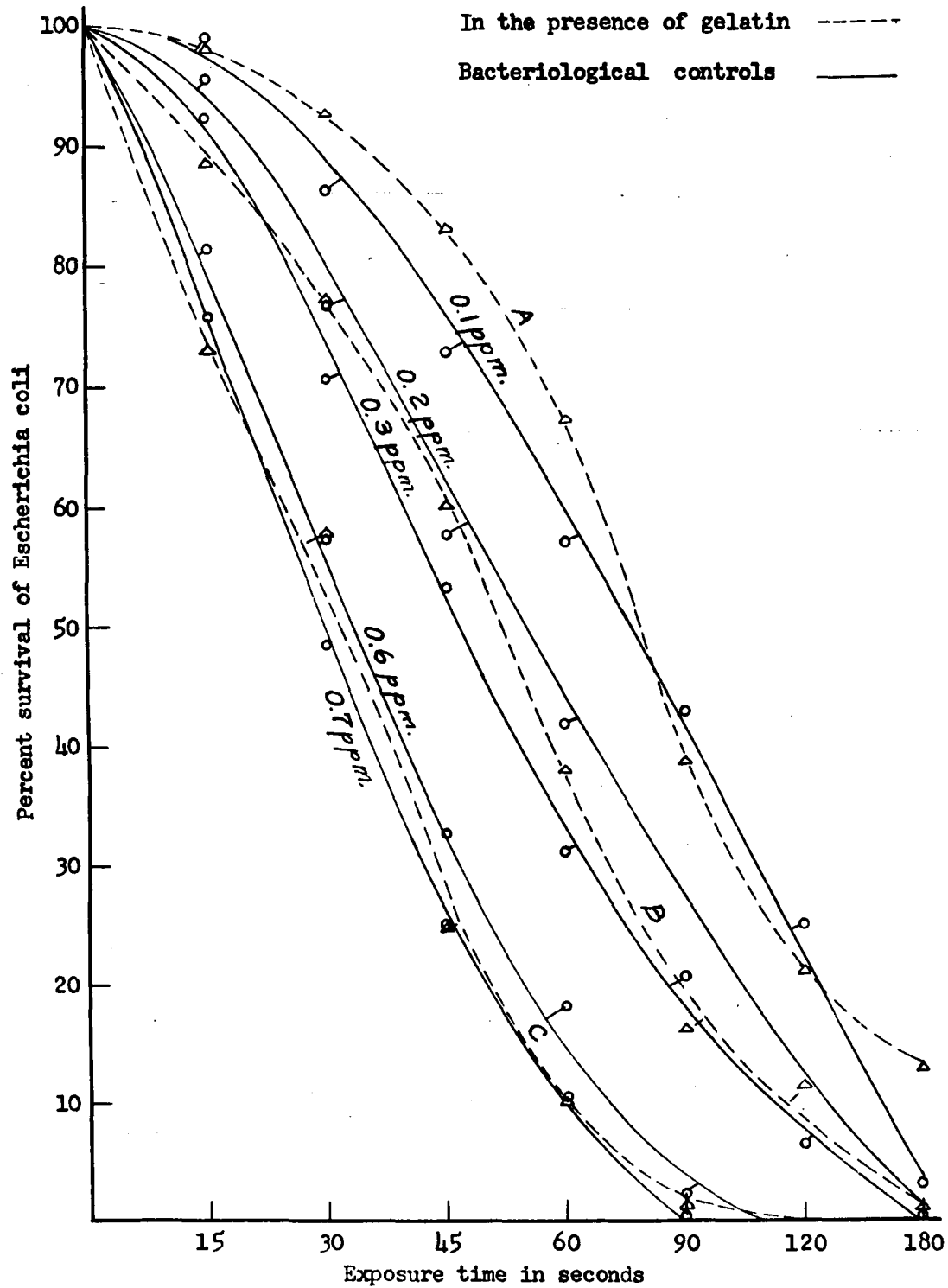
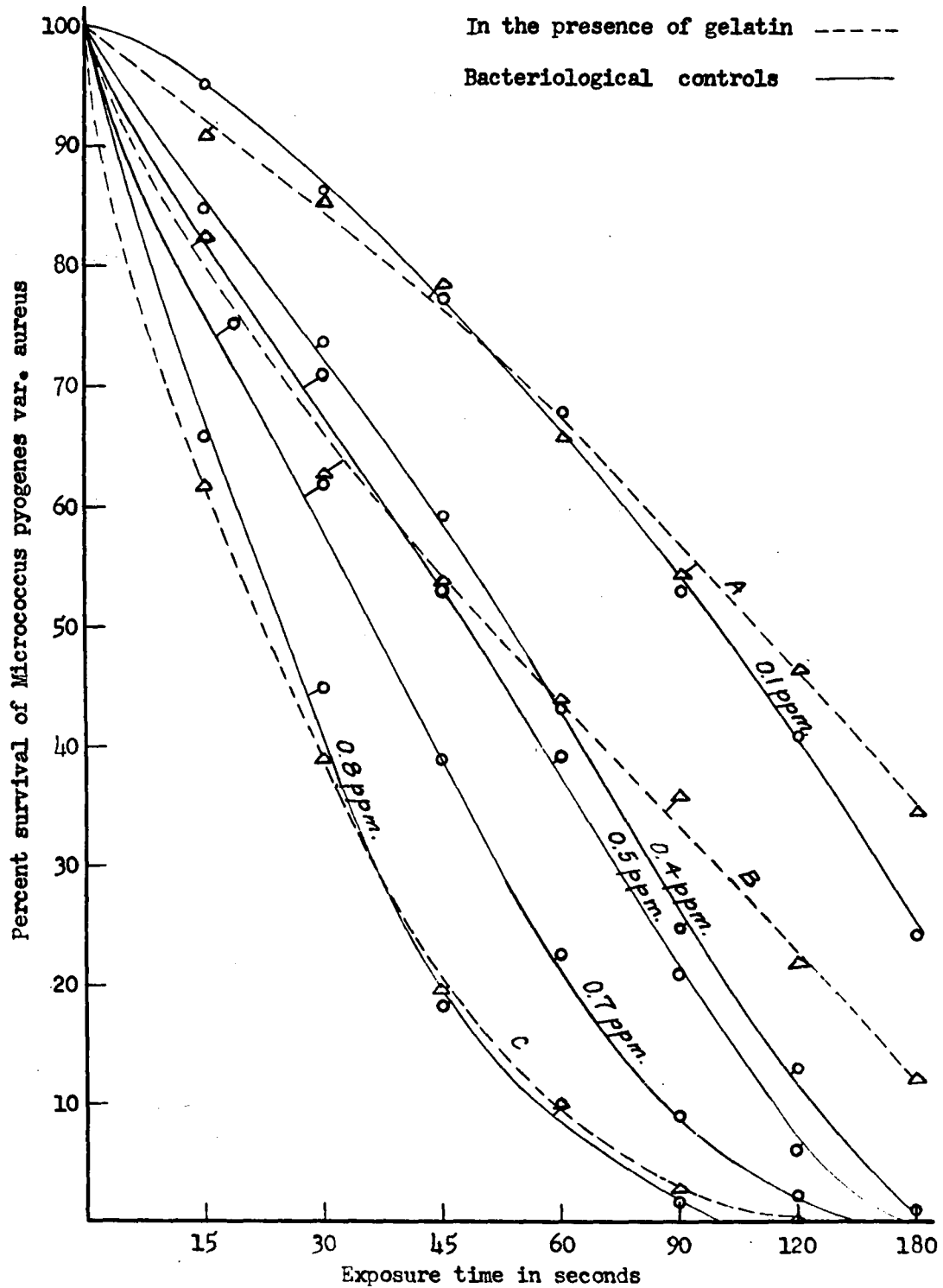


FIGURE VIII

The germicidal activity of various concentrations of chlorine
in the presence of 0.05 percent gelatin at pH 7.0 ± 0.1 and
 $25.0 \pm 1.0^\circ \text{C}$.



times higher than those obtained in germicidal tests; and the amperometric test failed to indicate any free or germicidal chlorine.

Although the results for the free chlorine tests varied for the three chemical tests, the general picture for gelatin was not unlike that obtained with agar-agar.

The next series of tests were made with suspensoids. In the first series of tests a finely divided activated charcoal was added at the rate of 0.025 gram per flask. Chlorine was added to the flasks in concentrations of 8.0, 15.0 and 20.0 ppm. The tests were run as usual.

The data are presented in Table VI and Figures IX and X. The bacterial titration showed approximately 0.20 to 0.30 ppm. of germicidal chlorine in Flask B. The chemical tests showed 0.65 ppm. for ortho-tolidine flash and ortho-tolidine arsenite tests and 0.20 ppm. for the amperometric method. Here again the chemical tests show approximately three times the amount of active chlorine obtained by bacterial titration. The amperometric test gave approximately the same picture as the bacterial method.

It is interesting to note that the amperometric test gave lower chlorine residuals than the ortho-tolidine flash and the ortho-tolidine arsenite tests on both gelatin and activated charcoal.

TABLE VI

The Germicidal Activity of Various Concentrations of Chlorine
in the Presence of 0.025 Grams of Charcoal in 500 Ml. Water at
pH 7.0 ± 0.1 and $25.0 \pm 1.0^{\circ}$ C.

Chemical titrations of chlorine									
No. of Trls.	Flask No.	PPM. Cl.	S.I. test	O. T. test		O. T. A. test		Amperometric test	
			total	free	comb.	free	comb.	free	comb.
10	A	8	1.20	0.20	0.95	0.20	0.96	0	1.00
11	B	15	2.75	0.65	1.98	0.65	1.98	0.20	2.30
11	C	20	6.40	1.50	4.05	1.50	4.00	0.35	4.55

No. of Trls.	Flask No.	Bacteriological test							
		Exposure time in seconds							
		0	15	30	45	60	90	120	180

Percent survival of <i>Escherichia coli</i>									
5	A	100	97.6	95.2	86.6	85.8	77.3	70.0	44.8
6	B	100	87.0	65.9	55.5	40.3	28.1	19.9	11.7
6	C	100	47.0	23.6	9.2	2.6	2.0	0.9	0

Percent survival of <i>Micrococcus pyogenes</i> var. <i>aureus</i>									
5	A	100	94.6	92.3	84.7	78.4	68.6	62.7	50.1
5	B	100	91.4	80.2	74.4	64.7	53.1	41.5	19.8
5	C	100	51.6	27.2	16.5	10.8	5.7	2.3	0.6

Bacteriological controls on Tables I & II.

FIGURE IX

The germicidal activity of various concentrations of chlorine in the presence of 0.025 grams of charcoal in 500 ml. water at pH 7.0 ± 0.1 and $25.0 \pm 1.0^\circ \text{C}$.

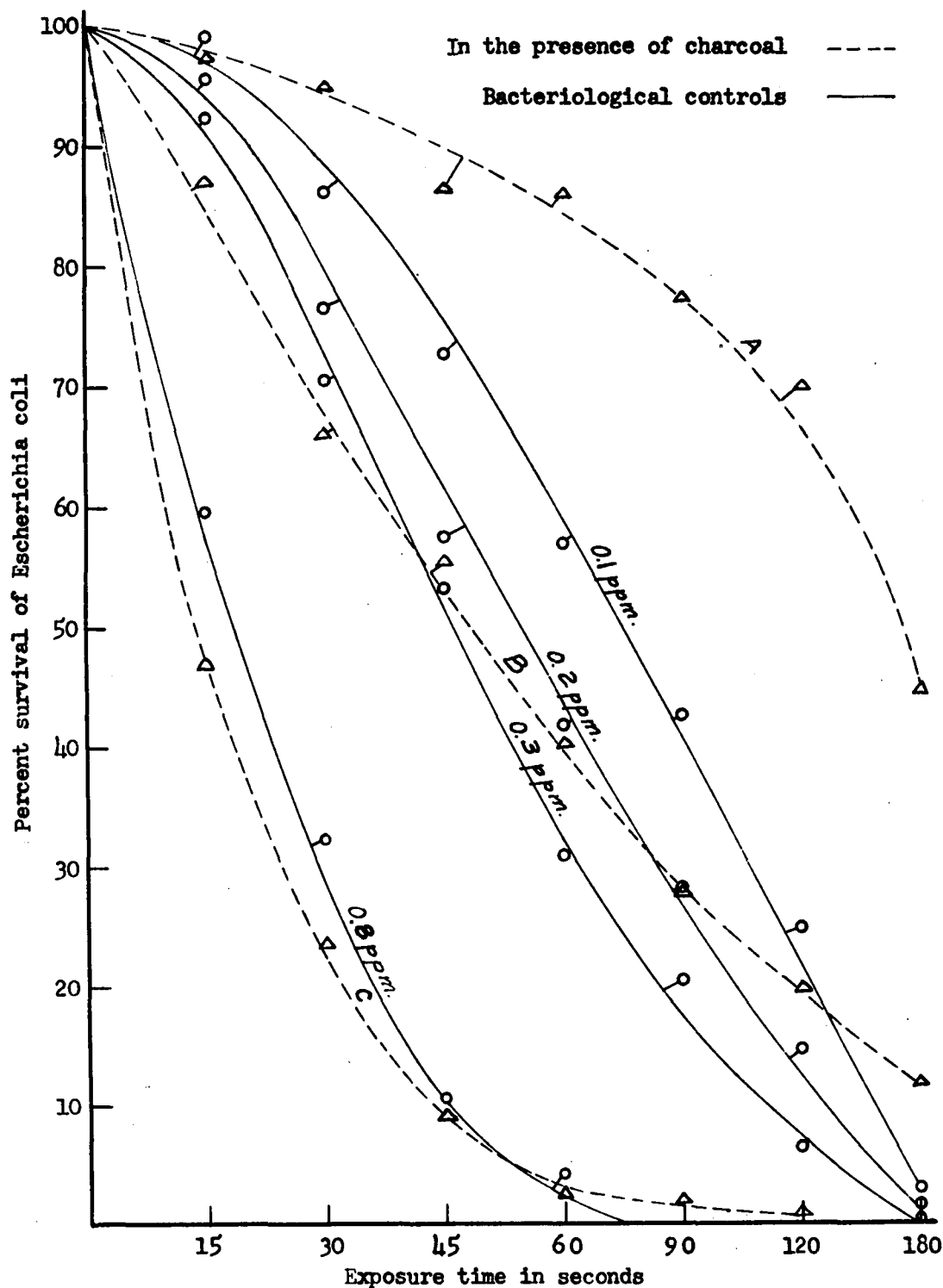
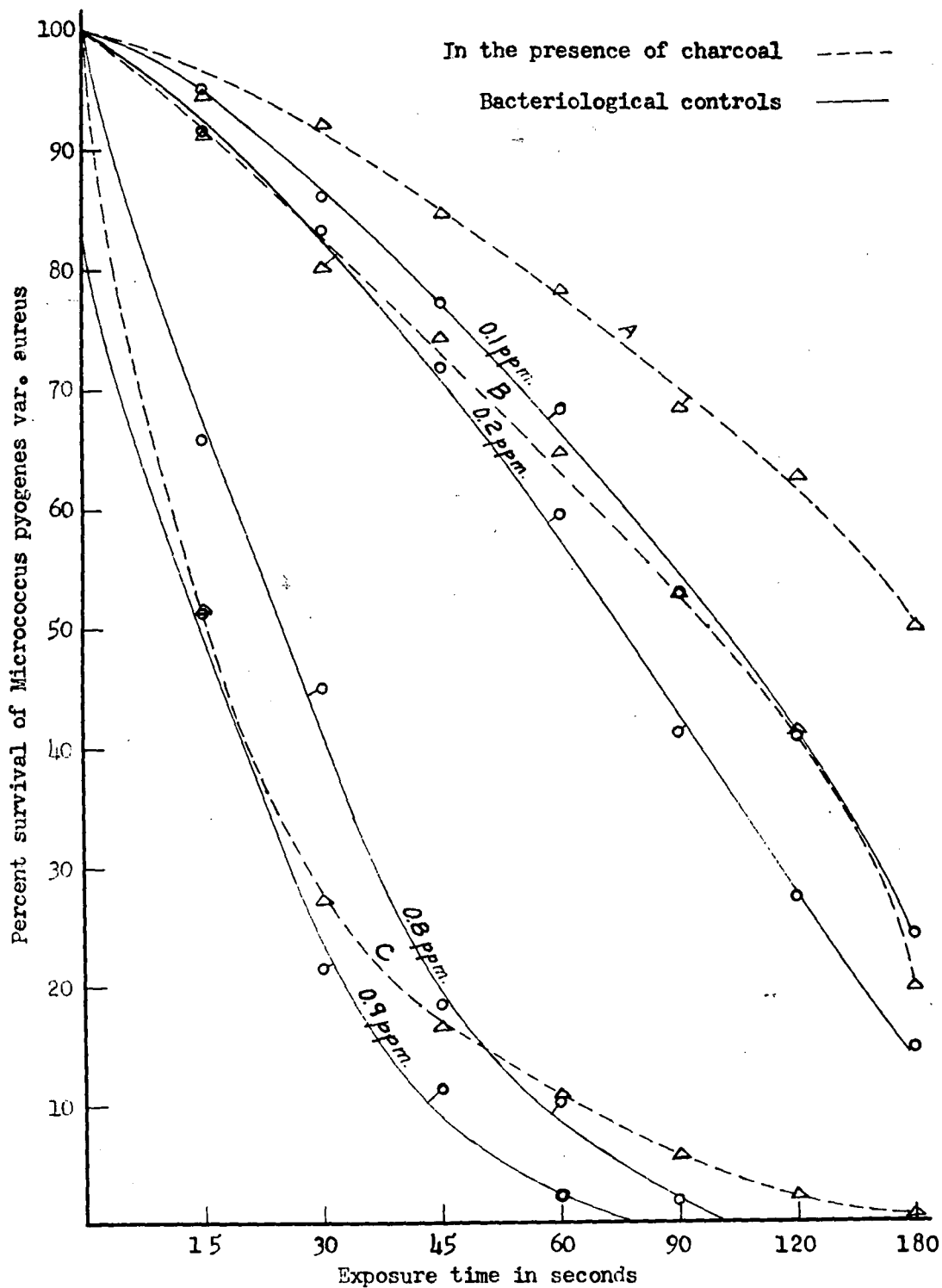


FIGURE X

The germicidal activity of various concentrations of chlorine in the presence of 0.025 grams of charcoal in 500 ML. water at pH 7.0 ± 0.1 and $25.0 \pm 1.0^\circ \text{C}$.



These data also confirm the observations of Mallmann and Edwards (14) on activated charcoal.

A second insoluble substance, diatomaceous earth was tested. This material was added at the rate of 0.01 percent to the flasks. Chlorine was added at the rates of 6.0, 10.0 and 20.0 ppm. The tests were made in the same manner as before.

The results are presented in Table VII and in Figures XI and XII. The bacterial titrations for Flask A showed, approximately 0.75 ppm. of germicidal chlorine for both Esch. coli and M. pyogenes var. aureus. The chemical titrations for Flask A showed 0.80 ppm. chlorine for the ortho-tolidine flash and the ortho-tolidine arsenite tests and 0.72 ppm. for the amperometric method.

With this substance, the chemical tests for free chlorine and the bacterial titration were similar. It would appear that the diatomaceous earth did not adsorb the free chlorine; thus, the germicidal chlorine and the free chlorine tests gave the same results.

For the last series of interfering substances skim milk was selected as a mixture of soluble and insoluble organic materials.

Skim milk, used in this experiment, was taken from

TABLE VII

The Germicidal Activity of Various Concentrations of Chlorine
in the Presence of 0.01 Percent Diatomaceous Earth at pH 7.0±
0.1 and 25.0 ± 1.0° C.

Chemical titration of chlorine									
No. of Trls.	Flask No.	PPM. Cl.	S. I. test	O. T. test		O. T. A. test		Amperometric test	
			total	free	comb.	free	comb.	free	comb.
11	A	6	1.55	0.80	0.55	0.80	0.55	0.72	0.51
7	B	10	4.78	4.00	0.50	4.00	0.50	4.40	0.50
7	C	20	14.80	13.00	0.60	13.00	0.60	12.50	0.55

No. of Trls.	Flask No.	Bacteriological test							
		Exposure time in seconds							
		0	15	30	45	60	90	120	180

Percent survival of Escherichia coli									
5	A	100	63.4	47.0	15.1	6.4	0.3	0	0
4	B	100	0	0	0	0	0	0	0
4	C	100	0	0	0	0	0	0	0

Percent survival of Micrococcus pyogenes var. aureus									
6	A	100	73.7	66.1	46.0	28.2	11.8	8.7	0
3	B	100	0	0	0	0	0	0	0
3	C	100	0	0	0	0	0	0	0

Bacteriological controls on Tables I & II.

FIGURE XI

The germicidal activity of various concentrations of chlorine
in the presence of 0.01 percent diatomaceous earth at pH 7.0±
0.1 and 25.0±1.0° C.

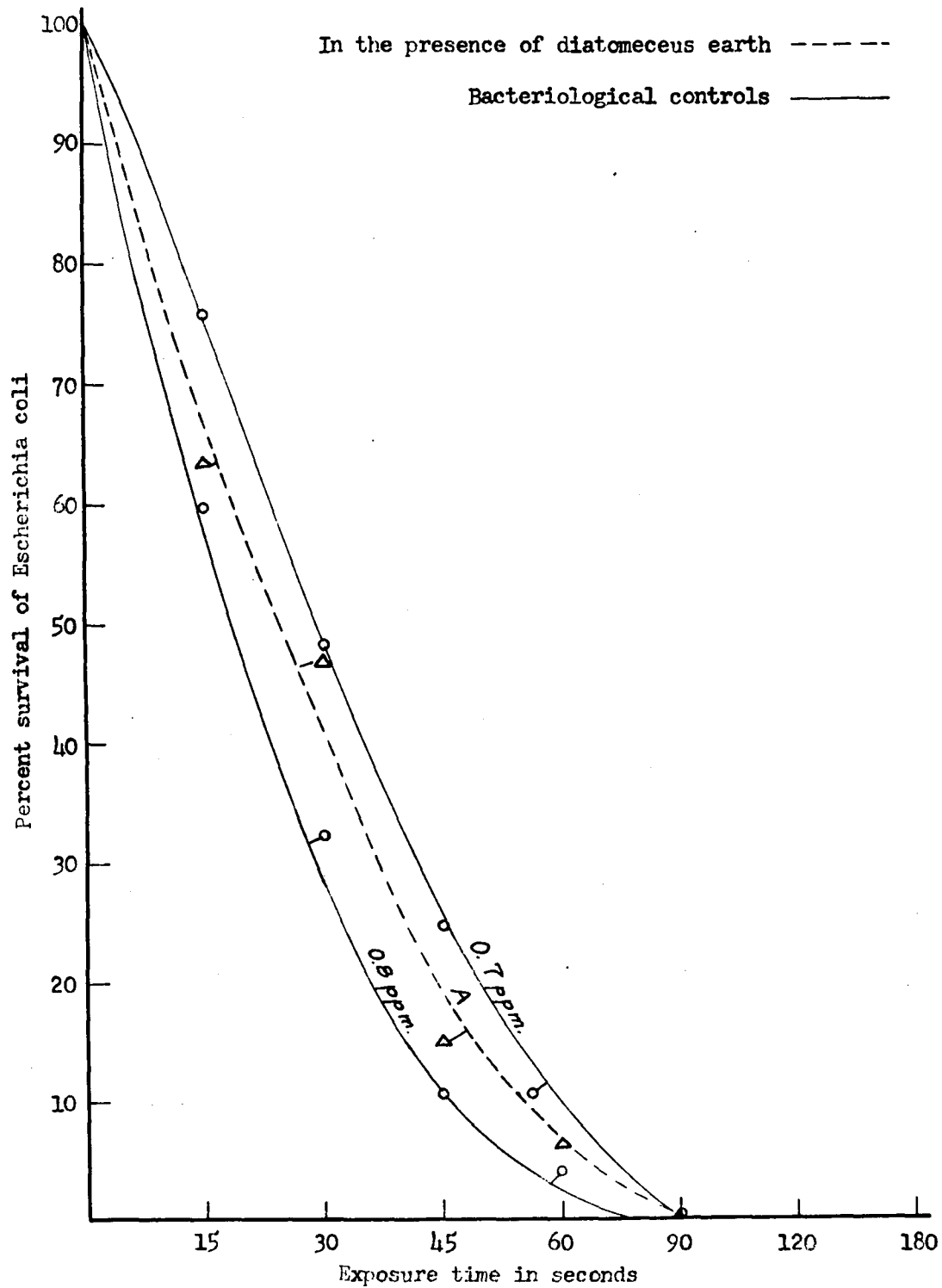
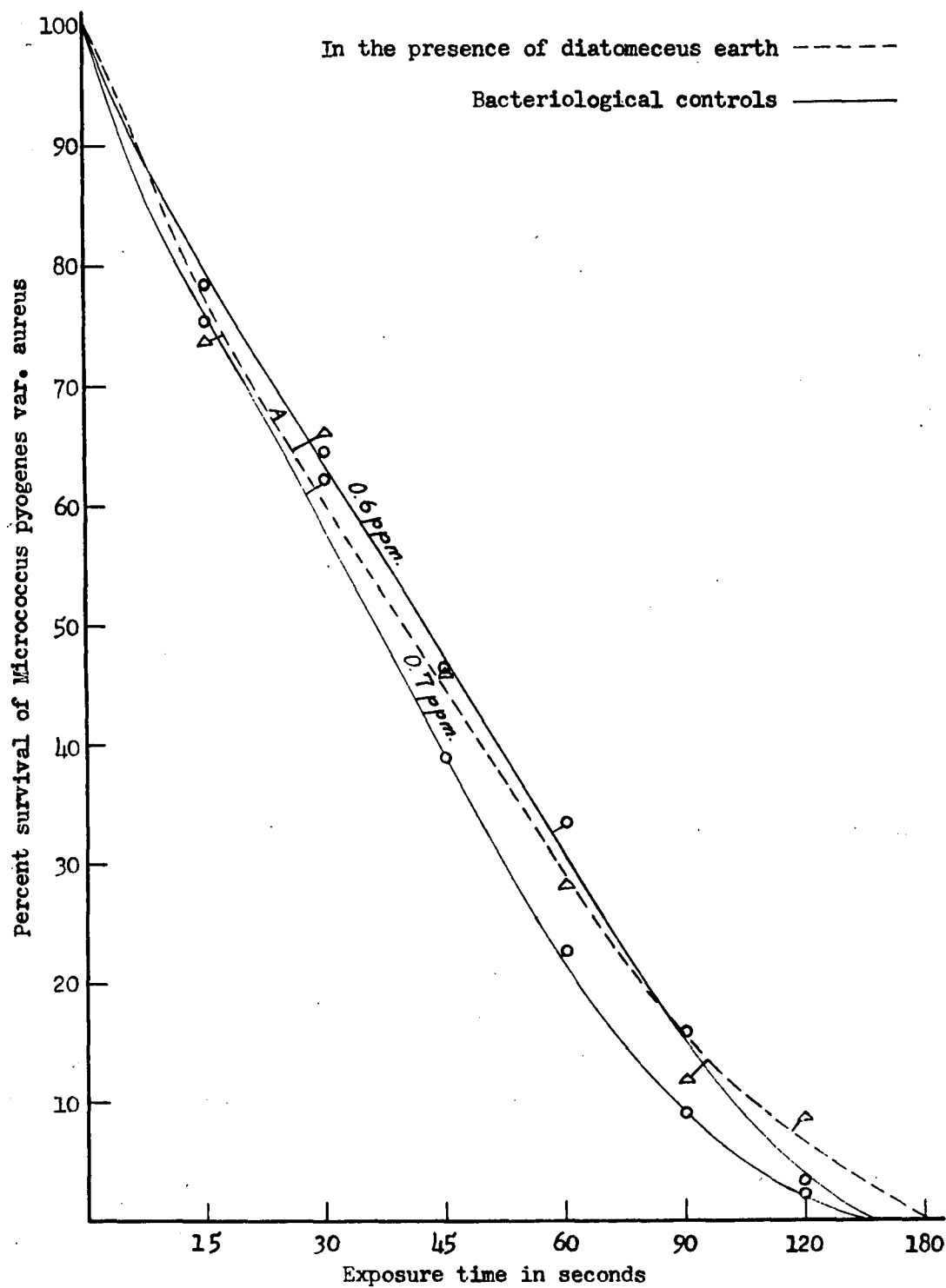


FIGURE XII

The germicidal activity of various concentrations of chlorine
in the presence of 0.01 percent diatomaceous earth at pH 7.0±
0.1 and 25.0±1.0° C.



one batch and sterilized in the autoclave in 5 ml. portions in tightly plugged test tubes which were stored in a refrigerator throughout the work. Sterile skim milk was added to 500 ml. sterile distilled, zero chlorine-demand water in a final concentration of 0.2 percent. To these flasks chlorine was added so that the final concentrations were 6.0, 8.0, 10.0, 15.0 and 20.0 ppm. The experiments were conducted as before.

The results are presented in Table VIII and Figures XIII and XIV. The bacterial titration for Flask C showed about 0.20 to 0.30 ppm. for Esch. coli, and about 0.20 ppm. of germicidal chlorine for M. pyogenes var. aureus. The chemical titrations for Flask C showed 0.45 ppm. free chlorine for the ortho-tolidine flash and the ortho-tolidine arsenite tests and 0.07 ppm. for the amperometric method. Here again the chemical tests showed approximately 1.5 to 2 times more active chlorine than the germicidal chlorine indicated by the bacteriological test. The amperometric test again failed to measure the actual amount of the free chlorine.

These data also confirm the observations of Mallmann and Edwards (14) for skim milk measured with the starch-iodide and the ortho-tolidine tests.

These experiments, involving the use of suspended

TABLE VIII

The Germicidal Activity of Various Concentrations of Chlorine
in the Presence of 0.2 Percent Skim Milk at pH 7.0 ± 0.1 and
 $25.0 \pm 1.0^{\circ}$ C.

Chemical titration of chlorine									
No. of Trls.	Flask No.	PPM. Cl.	S. I. test	O. T. test		O. T. A. test		Amperometric test	
			total	free	comb.	free	comb.	free	comb.
8	A	6	0.98	0.03	1.05	0.03	1.05	0	1.00
9	B	8	1.30	0.25	1.16	0.25	1.17	0	1.00
10	C	10	1.70	0.45	1.45	0.45	1.45	0.07	2.12
8	D	15	6.07	0.71	4.54	0.71	4.53	0.23	4.17
6	E	20	11.05	1.58	9.76	1.59	9.76	0.87	9.25

No. of Trls.	Flask No.	Bacteriological test							
		Exposure time in seconds							
		0	15	30	45	60	90	120	180

Percent survival of <i>Escherichia coli</i>									
4	A	100	99.7	93.4	91.0	89.1	82.9	68.2	52.8
5	B	100	97.3	88.4	81.3	72.0	61.1	51.5	34.6
5	C	100	90.2	75.9	59.0	43.3	33.0	24.8	11.5
4	D	100	74.2	54.2	37.9	19.1	6.7	0	0
3	E	100	25.9	8.6	2.1	0.2	0	0	0

Percent survival of <i>Micrococcus pyogenes</i> var. aureus									
4	A	100	99.6	98.0	97.8	97.0	95.1	93.0	89.2
4	B	100	98.0	88.1	81.0	70.2	60.6	50.5	32.0
5	C	100	96.9	85.8	77.0	63.3	50.0	36.0	20.3
4	D	100	94.3	77.5	45.8	33.2	23.0	8.2	0.6
3	E	100	77.0	58.5	39.5	16.8	8.0	1.4	0

Bacteriological controls on Tables I & II.

FIGURE XIII

The germicidal activity of various concentrations of chlorine in the presence of 0.2 percent skim milk at pH 7.0 ± 0.1 and $25.0 \pm 1.0^\circ \text{C}$.

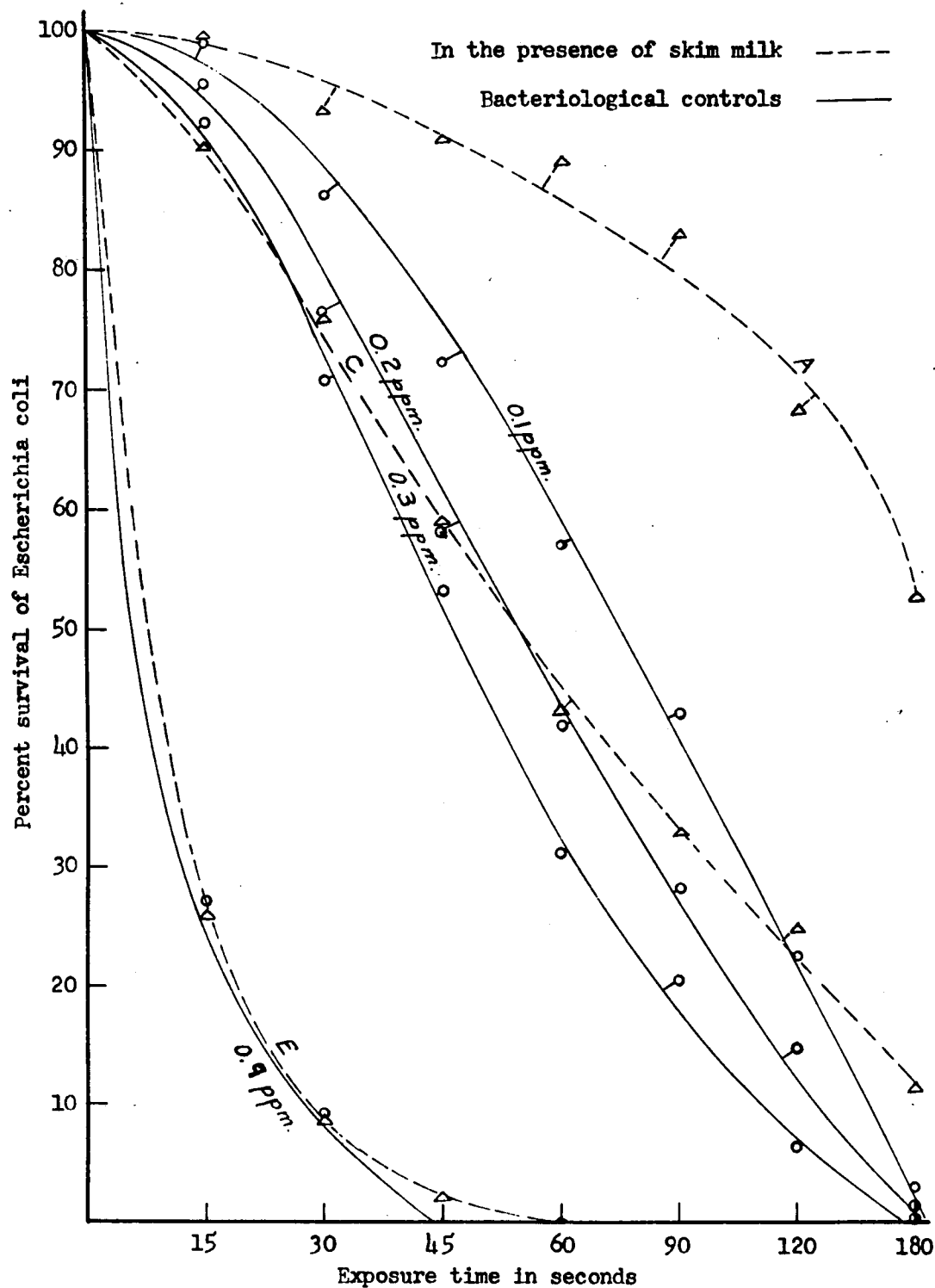
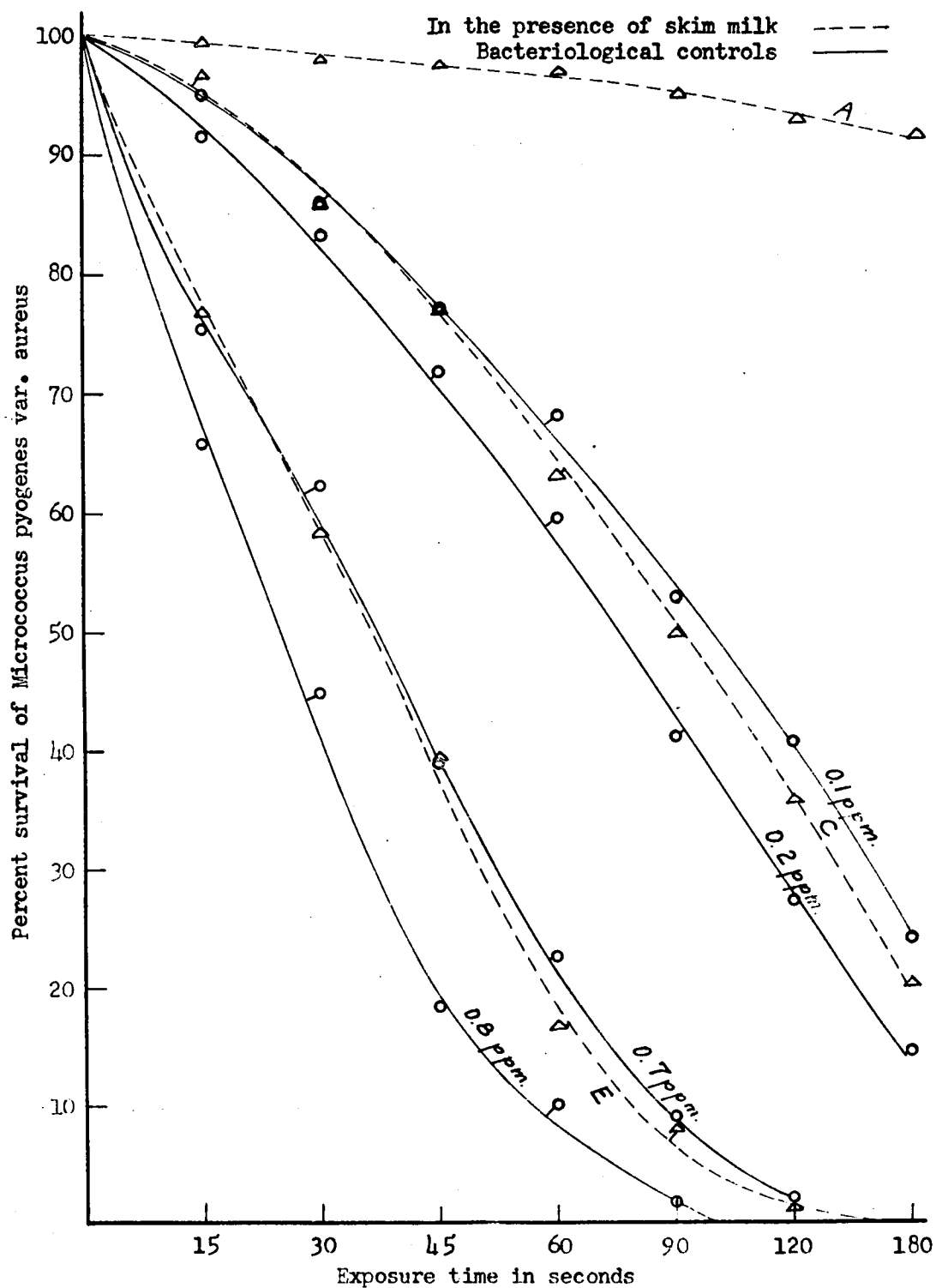


FIGURE XIV

The germicidal activity of various concentrations of chlorine
in the presence of 0.2 percent skim milk at pH 7.0 ± 0.1 and
 $25.0 \pm 1.0^\circ \text{C}$.



solids in the presence of chlorine showed that the chemical tests (ortho-tolidine flash, ortho-tolidine arsenite and amperometric methods) measure the adsorbed chlorine in addition to the free chlorine. Thus the so-called free chlorine actually represents both adsorbed and free chlorine and is not necessarily a measurement of the germicidal activity of the solution.

In the absence of suspended solids, in the absence or presence of soluble substances, the free chlorine, as measured by chemical titration, is identical to the germicidal chlorine titration obtained by the bacterial kill.

These data indicate that the discrepancies in the degree of bacterial kill and the chlorine residuals obtained in swimming pools by Mallmann and Seligmann (16) were likely due to the adsorbed chlorine present on the finely suspended particles in the swimming pool water.

It is also likely that in checking chlorine residuals in sewage effluents and rinse waters for dairy and food utensils, considerable variations might be obtained with the ortho-tolidine flash or the ortho-tolidine arsenite tests. How often such variations may result in false tests for measuring proper sanitation was not checked in the field.

The present classification of chlorine residuals

into two groups, namely combined and free, should be amended to include adsorbed when suspended solids are present even in minute quantities.

In any chlorine assay, where the chlorine solution contains suspended solids, acidulation of the solution causes a shift in the equilibrium between the free and adsorbed chlorine. The equilibrium would also be disturbed to a lesser degree if in the process of titration the chlorine was used up. Thus such tests would show higher chlorine residuals than the actual germicidal chlorine residuals.

Mallmann and Ardrey (13) demonstrated by bacterial kill titration, that the starch-iodide and the ortho-tolidine tests failed to measure the germicidal chlorine. They found that the oxidation-reduction potential method paralleled the bacterial kill titrations. This, they stated, was due to the fact that the potential between the electrodes in the solution did not in any sense disturb the chlorine equilibrium and measured only the chlorine available to react with bacteria.

At the last meeting of the American Public Health Association (1950) Dr. Wyllie of Queens College, Ontario reported on a new chlorine test using chymotrypsin as a titrating substance. Definite amounts of chymotrypsin

were added to the chlorine water. The chlorine combined with the chymotrypsin and rendered it inactive. Skim milk was then added to titrate the residual active chymotrypsin.

This method was reported after the writer had completed her laboratory work. There is every indication that this test will parallel the bacterial kill titration procedure.

SUMMARY

This work was done with the idea of determining the accuracy of various chemical methods by checking with bacteriological titrations in the presence and absence of soluble organic matter and suspended solids.

The starch-iodide, ortho-tolidine flash and ortho-tolidine arsenite tests were made according to the Standard Methods for the Examination of Water and Sewage (28, 29). All the solutions and reagents were prepared by the author from chemically pure substances. For the amperometric titration, the instructions for operating the instrument published by Wallace and Tiernan Co. (31) were followed very closely.

The method for the bacteriological test was a modified procedure adapted from one first proposed by Mallmann and Schalm (15).

In order to prevent errors due to external factors, pH, temperature, chlorine concentrations, the volume of solutions and the number of bacteria (within the possible limits) were kept constant.

It was observed that in the absence of organic and inorganic substances all the chemical tests and the bacteriological tests were equally accurate. However, in

the presence of suspended solids the accuracy of each chemical test depended more or less upon the substance introduced into the solution.

When dextrose was used, as an example of a soluble substance, both chemical and bacteriological titration methods gave identical results. Thus, the free chlorine, indicated by the ortho-tolidine flash test and amperometric method, and the germicidal chlorine, indicated by the bacterial kill titration, were the same.

When suspended solids, such as agar-agar, gelatin and charcoal, were introduced into the chlorine solution, the free chlorine obtained by chemical tests were far in excess of the germicidal chlorine.

In the presence of skim milk results were similar to those obtained with agar-agar, gelatin and charcoal.

When diatomaceous earth was used as the suspended solid the chemical tests for free chlorine and the bacterial kill method gave similar results. Thus it appears that the adsorption of the chlorine by suspended solids is selective.

CONCLUSIONS

1. The bacterial titration method can be used, at least as a research procedure, for the detection of germicidal chlorine, either in the presence or absence of organic and inorganic substances, in water.
2. Under standardized conditions the ortho-tolidine flash and the ortho-tolidine arsenite tests determine both the free and combined chlorine identically where no color interfering substances are present.
3. The germicidal chlorine values, obtained by the starch-iodide, ortho-tolidine flash or ortho-tolidine arsenite and the amperometric tests in the presence of organic and inorganic substances, are not in correlation with each other.
4. Soluble organic substances like dextrose, have little or no effect on the accuracy of the chemical and bacteriological tests in determining germicidal chlorine residuals, except in the case of the starch-iodide test, since this method measures only the total chlorine.
5. The free chlorine detected by the ortho-tolidine flash procedure in the presence of certain common colloids or suspensoids, measured both adsorbed and the germicidal chlorine.

6. The amperometric method showed no free chlorine in three instances, gelatin, charcoal and skim milk, when germicidal chlorine was present. In one instance, agar-agar, the free chlorine was in excess of the germicidal chlorine.

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