THE USE OF SURFACE-ACTIVE AGENTS AS A SOURCE OF CARBON BY THE COLIFORM GROUP

by

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INTRODUCTION

The microbiology of water has assumed great importance in public health, because water often has been a vector for transmission of food spoilage and pathogenic organisms. Pathogenic bacteria are very difficult to isolate from water with certainty in a reasonable length of time, hence, bacteriologists have devised indirect methods for detecting the possible presence of pathogens.

The standard procedure for detecting sewage pollution of water is to test water for the presence of "coliform" bacteria, and if they are present there is a good possibility that pathogenic enteric bacteria may also be present in the water. To the sanitarian the "coliform" group is subdivided into two significant genera, Escherichia and Aerobacter. The genus Escherichia is usually considered to be found in cases of "fecal" pollution; while the genus Aerobacter is of the "grain" type; i.e., found in cases of pollution from decomposing organic material. Since the genus Escherichia is considered to be of "fecal" origin, it should be differentiated from the "non-fecal" genus Aerobacter. At present there is no single, simple, specific test for the presence of Escherichia in the presence of other organisms. If it were possible to devise a simple test to distinguish Escherichia in the presence of Aerobacter species in a short period of time, it would greatly improve the methods for water analysis.

If it were possible to devise a test based on a different

concept; e. g., inhibition of "nonenterics" and promotion of growth of the "enterics", coupled with the use of some agents usable as the sole source of carbon only by the genus Escherichia, the procedure of water analysis would be greatly improved.

This investigation was proposed to test a series of surface-active agents to determine whether one could be found which would inhibit "nonenteric" species and could be used as a source of carbon by Escherichia species but not by Aerobacter species.

REVIEW OF THE LITERATURE

Synthetic surface-active agents are relatively new products in the field of industrial chemistry, hence, very few data have been published on their relation to bacteriology. Some material has been published on the bacteriostatic properties and the possible use of surface-active agents in the field of bacteriology.

Gershenfield and Milanick (1941) reported that the bacteriostatic action of surface-active agents depended upon the following factors: (1) whether the compound was anionic or cationic; (2) the hydrogen-ion concentration of the environment; and (3) the specific nature of the organism or organisms, whether Gram positive or Gram negative. The cationic depressants exhibited their greatest efficiency in the alkaline range, and the higher the pH the greater the efficiency. The anionic depressants exhibited their greatest efficiency in the acid range, therefore, the lower the pH the greater the efficiency.

Studies on the significance of hydrogen-ion concentration and the bactericidal efficiency of surface-active agents were made by Gershenfield and Perlstein (1941). They found that surface-active agents exert a marked increase in bactericidal efficiency with an increase in hydrogen-ion concentration.

Scales and Kemp (1941), on the other hand, used surface-active agents in an acid range and found that at pH 4.0 some of the surface-active agents were germicidally superior to alkaline sodium hypochlorite. A 0.01 percent concentration of surface-active agent at pH 4.0, with phosphoric acid as the

acidifier, could be employed successfully, but a 0.03 to 0.05 percent concentration at pH 4.0 was recommended. Phosphoric acid and gluconic acid were suggested as acidifiers because of their low corrosiveness. Bacterial endospores were found to be very resistant to the action of the surface-active agents, but a 30 minute exposure at 65.5°C. and pH 4.0 destroyed Bacillus subtilis spores. A temperature higher than room temperature increased the bactericidal effect of the surface-active agent.

Ordal and Deromedi (1943) reported that two of the wetting agents, sodium lauryl sulfonate and dioctyl ester of sodium sulfosuccinate, greatly enhanced the germicidal action of buffered solutions of phenolic compounds. This action is probably due to the synergistic action between the wetting agents and the undissociated phenolics.

That the "killing" action of surface-active cations on bacteria can be reversed under certain conditions was reported by DuBois and Valko (1944). The action can be reversed by detoxification with a high molecular anion. This can be considered as a phenomenon of ionic exchange occuring on the surface of the bacteria.

The nature of the bactericidal action of surface-active agents was studied by Hotchkiss (1946). Surface-active agents may combine with oppositely charged sites upon the bacterial surfaces. This process can be prevented or reversed through competition of such substances as phosphatides, other detergents, or hydrogen and hydroxyl ions. Adsorption of a small fraction of the maximum amount that can combine in some cases

results in irreversible damage to the cellular membrane, with accompanying release of nitrogen and phosphorus compounds from the cells. The cells can no longer repair themselves and autolyze at a rate and to an extent characteristic for each species and strain. The surface-active agent may denature the metabolic enzyme system. This type of cytolytic injury is not noticeably important in the killing of bacteria by other types of antiseptics.

While testing bactericidal detergents used for sanitizing eating utensils, Guiteras and Shapiro (1946) found that anionic agents are generally superior to both cationic and non-ionic agents as detergents. Cationic agents are superior as germicides; but when cation-active agents are used as bactericides in detergent compositions, it is essential that the detergent be emulsifying but not saponifying. By being saponifying the surface-active agents will form insoluble compounds and in that way will not be fully active as cations.

In explaining the bactericidal action of cations, DuBois and Dibblee (1947) reported that the surface-active cations must first combine with the surface-active anions on or in the bacteria, and these ions are then inactivated. The cations must first reach the surface-active anions of the bacteria which may have penetrated the bacteria to varying extents, and only then can the excess cations exert their antibacterial action.

Klein and Kardon (1947) reported that the activity of the cationic sanitizing agents "Zephiran" and "Phemerol" against Gram positive and Gram negative bacteria cannot be reversed by

the anionic detergents sodium decyl sulfate or sodium lauryl sulfate.

Glassman (1948) made a comprehensive review of the properties of surface-active agents and their relation to bacteriology. An anionic surface-active agent is characterized by a structural balance between a hydrophobic residue and a negatively charged hydrophilic group. Cationic surface-active agents have the same hydrophobic residues and may be balanced with a positively charged hydrophilic group. Nonionic surface-active agents possess no ionized groups. The hydrophobic portion of a nonionic surface-active agent is balanced by such nonionized hydrophilic groups as polymerized ethylene oxide or polyhydric alcohols. In media of low surface tension, Bacillus subtilis and Mycobacterium tuberculosis may cease pellicle formation and grow submerged and dispersed throughout the bulk of the liquid; under similar conditions some anaerobes, particularly Clostridium tetani, have been reported to grow "aerobically". Bacteria flourishing in the gastrointestinal tract seem to be resistant to the deleterious action of surface-active compounds in culture media.

In addition, cationic compounds possess equal effectiveness against Gram positive and Gram negative organisms, while the anionic compounds show a selective activity against Gram positive organisms. In general, the results of studies of inhibition of bacterial metabolism and bactericidal activity of surface-active agents parallel each other, although quantitative correlation has not always obtained. As one departs from a near neutral pH level, the cationic compounds tend to become more efficient in acid

solutions; the reverse situation holds true for the anionic compounds.

Cationic germicides were found to be readily adsorbed on glass surfaces by Migaki and McCullock (1949) and thus could be readily removed from solution. The surface and surface electrical charge of organisms exposed to cationic agents are known to undergo alteration.

An outstanding current application of surface-active agents in bacteriology is the promotion of submerged and diffuse growth of tubercle bacilli (Mycobacterium tuberculosis) by "Tween 80", a nonionic, fatty acid ester type compound. Although the virulent tubercle bacilli are among the least fastidious of pathogenic microorganisms, the in vitro growth of these organisms has necessitated large inocula and long periods of incubation.

Utilizing a modified Kirchner medium DuBos and Davis (1947) demonstrated that the addition of "Tween 80", up to an optimal level of 0.1 percent, greatly enhanced the rate and abundance of growth of the tubercle bacilli. While "Tween 80" promoted the submerged growth of tubercle bacilli, the experiments usually required relatively large inocula. This inhibitory effect against small inocula has been found to be due to small amounts of unesterified oleic acid present in the original "Tween 80" or formed as a hydrolytic product by the organisms. Extraction of this unesterified fatty acid, or its removal from the field of action by "complex" formation with such substances as serum albumin, allows successful growth from inocula of 2 or 3 cells.

Sattler and Youmans (1948) found "Tween 80" to be the most

suitable agent of those tested when added to synthetic culture media for promoting the growth of virulent tubercle bacilli. The advantages claimed were that this substance exerted a stimulating effect on growth and produced a diffuse homogenous growth of tubercle bacilli; the growth was homogenous and similar to that of many non-acid-fast bacteria, instead of the granular or flaky growth usually encountered. Commercial preparations of "Tween 80", however, contained a sufficient amount of unesterified oleic acid to exert a growth-inhibitory effect on small inocula of tubercle bacilli. Unpurified "Tween 80" markedly inhibited the growth rate of virulent tubercle bacilli in Proskauer and Beck synthetic medium, while glucose and glycerol stimulated the rate of growth.

DuBos, Davis, Middlebrook, and Pierce (1946) reported that the tubercle bacilli grown in submerged liquid culture in the presence of "Tween 80" retain their morphologic and staining characteristics. This has been true even in cultures maintained for over a year with repeated transfers in liquid media containing this product. Return of these cultures at any time to standard media causes reversion to a granular type of growth. In addition to the retention of morphologic and staining characteristics, pathogenic mycobacteria grown in the presence of "Tween 80" are extremely virulent for mice, guinea pigs, and chick embryos, and are able to facilitate the production of agglutinins for the homologous culture. It is well to caution, however, that "Tween 80" in conjunction with the tubercle bacilli may act as a haptene, thus serving as a potential source of confusion in immunologic analysis.

In 1948 Youmans and Youmans reported that the presence of "Tween 80" in synthetic media increased the bacteriostatic action of penicillin, streptomycin, and subtilin on the subsurface growth of the tubercle bacilli. "Tween 80" in a synthetic medium, as compared to synthetic media alone, increased the tuberculostatic activity of 15 out of 20 compounds.

Numerous other genera and species of bacteria have been studied in relation to surface-active agents.

Stimulated by the obvious advantages of having a water-soluble, non-toxic solution of oleic acid in the form of "Tween 80", Williams et al. (1923) have made use of this compound in studying the metabolism of lactic acid bacteria.

Another application to bacteriology of surface-active agents was discovered by Harris and McClure (1942). They found that Duponol WA flakes in a 0.2 percent solution has the same activity as 1 percent sodium desoxycholate in solubility tests for differentiating pneumococci from other microorganisms producing greenish discoloration of blood agar.

Surface-active agents also have an effect on phagocytosis. Berry et al. (1948) reported that 5 surface-active agents, out of a total of 52 tested <u>in vitro</u>, doubled the mean number of bacteria ingested per human neutrophile as compared to control values.

An organism resembling the genus <u>Pseudomonas</u> was isolated by Williams and Ress (1950) from a commercial shampoo. This organism fermented only glucose (feebly); grew on solid media with ammonium lactate or sodium asparaginate as sole source of nitrogen and carbon; and grew with ammonium phosphate as a nitrogen source when

either glucose or citrate served as a carbon source. Inferior growth of the organism was obtained on 5 percent sodium lauryl sulfate agar with or without ammonium phosphate added. In the preliminary report Williams and Ress mentioned that two (unnamed) anionic surface-active agents would support the growth of some types of bacteria.

EXPERIMENTAL MATERIALS

The idea of devising a new test based on the concept of inhibition of "nonenterics" and the use of surface-active agents as the source of carbon by the genus Escherichia in the presence of the genus Aerobacter was explored in this work.

Surface-Active Agents. The surface-active agents used in this investigation were secured from American, commercial producers of surface-active agents. Tables 1, 2, and 3 list the name, manufacturer, and composition of the agents used in this investigation.

Table 1. The cationic surface-active agents employed.

Trade name	Manufacturer	Chemical description
Ammonyx T	Onyx Oil and Chemical	Cetyl dimethyl benzyl ammonium chloride
DuPont retarder Lan	E. I. duPont de Nemours	Stearyl trimethyl ammonium bromide
Emcol E-607L	Emcol Corporation	Lauryl colamino methyl pyridinium chloride
Lauryl pyridinium chloride	Hooker Electrochemical	Same as trade name
Octab	Fairfield Laboratory	Octadecyl-dimethyl-benzyl ammonium chloride
Roccal	Winthrop-Stearns	Alkyl-dimethyl-benzyl-ammonium chloride

Table 2. The anionic surface-active agents employed.

Trade name	Manufacturer	Chemical description
Arctic Syntex A	Colgate-Palmolive-Peet	Fatty acid sulfonate
Aerosol OT	American Cyanamid	Dioctyl sodium sulfo-succinate
Alkanol B	E. I. duPont de Nemours	Alkyl-naphthalene sulfonate
Aresklene 400	Monsanto Chemical	Dibutyl-phenylphenol sodium disulfonate
Drene	Proctor-Gamble	Tri-ethanolamine alkyl sulfate
Duponol LS Flakes	E. I. duPont de Nemours	Sodium salt of sulfated ocenol fatty alcohol with added sodium sulfate
Duponol WA paste and flakes	Same	Sodium lauryl sulfate
Igepon AP	General Dyestuff	Sodium sulfonate compound of the oleic ester of an aliphatic compound
Igepon T	Same	Sodium salt of amide of oleic acid and methyl taurine
Igepal CA	Same	Polymerized ethylene oxide
Santomerse 3	Monsanto Chemical	Dodecyl-benzene sulfonate
Tergitol 4	Carbide and Carbon	7-ethyl 2-methyl undecanol-4
Tergitol 7	Same	Sodium sulfate derivative of 3, 9-diethyl tridecanol-6
Tergitol 8	Same	Sodium sulphate derivative of 2-ethyl hexanol-1

Table 2 (concl.).

Trade name	Manufacturer	Chemical description
Triton X-400	Rohm and Haas	Alkyl aryl ether sulfate
Triton W-30	Same	Sodium salt alkylated aryl ether sulphate
Victawet 58-B	Victor Chemical	Phosphorated capryl alcohol

Table 3. The non-ionic surface-active agents employed.

Trade name	Manufacturer	Chemical description
Carbowax 1500 dioleate	Glyco Products	Oleic acid ester of a polymerized polyethylene glycol
Polyethylene glycol 400 monooleate	Same	Oleic acid ester of a polymerized polyethylene glycol
Span 80	Atlas Powder	Sorbitan monooleate
Tween 80	Same	Polyoxyethylene sorbitan monooleate

Cultures. Nine of the "coliform" organisms were isolated from common sources and the remaining 28 were obtained from the stock culture collection of Dr. T. H. Lord. The 37 cultures were first checked for purity and determinative tests were made to determine the genus and species as outlined by the Manual of Methods for Pure Culture Study of Bacteria and the names were determined by use of Bergeys' Manual of Determinative Bacteriology, 6th. edition. It was found that 15 of the cultures were of the genus Escherichia and 22 of the cultures were of the genus Aerobacter. Table 4, which follows, lists the numbers of the cultures used and their genus and species.

Table 4. Names of organisms

Organism number	Organism name *
1	Escherichia acidilactici
2	Escherichia coli
3	Escherichia coli
4	Escherichia acidilactici
5	Escherichia coli
6	Escherichia coli
7	Escherichia coli
8	Escherichia coli
10	Aerobacter aerogenes
13	Escherichia acidilactici
15	Aerobacter aerogenes
18	Aerobacter aerogenes
19	Aerobacter aerogenes
47	Aerobacter cloacae
52	Aerobacter aerogenes
55	Aerobacter cloacae
59	Aerobacter aerogenes
66	Aerobacter aerogenes
68	Aerobacter cloacae
70	Aerobacter cloacae
76	Aerobacter aerogenes
80	Aerobacter aerogenes
82	Aerobacter aerogenes
84	Escherichia freundii
85	Aerobacter cloacae
87	Escherichia freundii
91	Aerobacter aerogenes
92	Escherichia coli
93	Aerobacter aerogenes
96	Aerobacter aerogenes
97	Escherichia intermedium
104	Aerobacter aerogenes
109	Aerobacter cloacae
140	Aerobacter aerogenes
141	Escherichia coli
145	Aerobacter aerogenes
154	Escherichia intermedium

* All names of organisms are in conformance with Bergey's Manual of Determinative Bacteriology, 6th Edition, 1948.

EXPERIMENT I

Determination of Toxicity of Surface-Active Agents on "Coliform" Bacilli

Before any surface-active agent could be employed in the prospected experiment its toxicity to the test organisms in the medium employed had to be ascertained. By adding the surface-active agents to a basic medium in different concentrations, the smallest amount of surface-active agents that was toxic to the different organisms could be ascertained. The toxicity of each surface-active agent against each organism had to be known, because the basis of the final test to be devised was the evidence of growth. Hence, the organisms could not be added to a toxic level of the surface-active agent or they could not grow. The 37 cultures of "coliform" bacteria were then subjected to varying amounts of the surface-active agents to find the smallest amount of agent that was toxic to the different cultures.

Procedure. Standard nutrient broth, which consisted of Bacto-peptone ten grams, sodium chloride five grams, Bacto-beef extract three grams, and water 1000 milliliters, adjusted to pH 7.0 served as the basic medium to which the varying amounts of surface-active agents were added. Ten-fold dilutions of the agents from one to ten (1-10) up to one to one million (1-1,000,000) in terms of the final medium, were used in this experiment. Nutrient broth cultures of the organisms were prepared and incubated for 24 hours at 37°C. These were used as the inoculum. The amount of inoculum used in each case was one loopful, the loop being a 20 gauge Nichrome wire loop 4 millimeters in diameter.

The different dilutions were inoculated and incubated for 48 hours at 37°C. After incubation the tubes were observed for growth, which constituted a positive reaction. Some of the media containing the lower dilutions of the surface-active agents were either too turbid or precipitated out, hence, the lowest dilution that could be read was used as the lowest dilution of toxicity.

Results. Some of the surface-active agents were either too turbid or precipitated out, consequently, the lowest dilution in which growth could be observed was recorded as the toxic level. The toxic level of the "coliform" organisms when subjected to each of the surface-active agents did not vary, in fact, the level of toxicity for each surface-active agent was the same with all organisms. Names of the cultures appeared on Table 4.

The lowest dilution of cationic surface-active agents in standard nutrient broth, in which growth of cultures 1 to 154 inclusive, was observed, is as follows:

Ammonyx T.	1-10,000
DuPont Retarder Lan.	1-10
Emcol E-607L	1-10,000
Lauryl Pyridinium chloride . . .	1-1,000
Octab.	1-10,000
Roccal	1-10,000

The lowest dilution of anionic surface-active agents in standard nutrient broth, in which growth of cultures 1 to 154 inclusive, was observed, is as follows:

Aerosol OT	1-100[1]
Alkanol B	1-10
Aresklene 400	1-10
Arctic Syntex-A	1-10
Drene	1-10
Duponol LS Flakes	1-100[2]
Duponol WA Paste and Flakes	1-10
Igepon AP	1-10
Igepon CA	1-100
Igepon T	1-10
Santomerse 3	1-10
Tergitol 4	1-10
Tergitol 7	1-10
Tergitol 8	1-10
Triton X-400	1-1,000[3]
Triton W-30	1-1,000[4]
Victawet 58-B	1-100

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[1] Note--Aerosol OT was too turbid to read in a 1-10 dilution.
[2] Note--Medium with Duponol LS Flakes was originally too turbid in 1-10 dilution to be used.
[3] Note--Triton X-400 was precipitated in 1-10 and 1-100.
[4] Note--Triton W-30 was originally turbid in 1-10 and 1-100.

The lowest dilution of non-ionic surface-active agents in standard nutrient broth, in which growth of cultures 1 to 154 inclusive, was observed, is as follows:

Carbowax 1500 Di-Oleate.	1-1,000[1]
Polyethylene Glycol 400 Monooleate .	1-1,000[2]
Span 80.	1-10
Tween 80	1-10

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[1] Note--Media with Carbowax 1500 Di-Oleate were originally turbid in 1-10 and 1-100.

[2] Note--Media with Polyethylene Glycol 400 Monooleate were originally turbid in 1-10 and 1-100.

EXPERIMENT II

Surface-Active Agents as a Source of Carbon for "Coliform" Bacilli

After finding the toxicity level of each of the surface-active agents for each organism, it was then possible to check each surface-active agent to see if it could be utilized as a sole source of carbon by each organism.

Media. The medium used in this experiment was patterned after Koser's citrate medium which is used in studying "coliform" organisms. The medium consisted of sodium ammonium phosphate 1.5 grams, monopotassium phosphate 1.0 grams, magnesium sulphate 0.2 grams, and the surface-active agent (substituted for the sodium citrate) 3 grams. It was hoped that the genus Escherichia would be able to utilize some surface-active agent as the sole source of carbon while the genus Aerobacter would not be able to utilize the same agent as the sole source of carbon. If this were possible it would be relatively easy to differentiate between the two genera.

Procedure. The basic medium was made up and the surface-active agents were then added. The solutions were adjusted to pH 7.0, tubed in 5 ml amounts, and sterilized by autoclaving at 121°C. for 15 minutes. Standard nutrient broth cultures of the organisms were prepared and incubated for 24 hours at 37°C., and then used as the inoculum. The tubes of the surface-active agent solutions were then inoculated with one loopful of the broth cultures of the organisms, the loop being a 20 gauge Nichrome wire loop 4 mm in diameter. All cultures were incubated for 24 hours

at 37°C. Growth after this period of time was determined by noting any visible turbidity. Any amount of growth was considered a positive test.

Results. The results of the test are divided into the two genera so as to facilitate interpretation. The surface-active agents are listed alphabetically. The results of the Escherichia organisms being subjected to the different surface-active agents are expressed in Table 5. The results of the Aerobacter organisms being subjected to the different surface-active agents are expressed in Table 6.

Table 5. The presence of growth of the Escherichia organisms in a 0.3 percent solution of each of the surface-active agents in the basic medium.

Culture number	Surface-active agents																							
	Aerosol OT	Alkanol B	Aresklene 400	Arctic Syntex-A Powder	Carbowax 1500 Di-Oleate	Drene	Duponol WA Flakes	Duponol WA Paste	Duponol LS Flakes	DuPont Retarder Lan	Igepon AP	Igepon T	Igepal CA	Lauryl Pyridinium Chloride	Polyethyl-ene Glycol Monooleate	Santomerse 3	Span 80	Tergitol 4	Tergitol 7	Tergitol 8	Triton W-30	Triton X-400	Tween 80	Victawet 58-B*
1	yes	yes	no	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	yes	no	yes	
2	no	yes	no	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	yes	no	yes	
3	no	yes	yes	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	yes	no	yes	
4	no	yes	yes	yes	no	no	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	yes	no	yes	
5	no	yes	no	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	yes	no	yes	
6	no	yes	yes	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	yes	no	yes	
7	no	yes	no	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	yes	no	yes	
8	no	yes	no	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	yes	no	yes	
13	no	yes	no	yes	no	no	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	yes	no	yes	
84	no	yes	yes	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	no	no	yes	
87	no	yes	yes	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	yes	no	yes	
92	no	yes	no	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	yes	no	yes	
97	no	yes	no	yes	no	no	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	no	no	yes	
141	yes	yes	yes	yes	no	no	no	no	no	no	no	no	no	no	no	no	yes	no	no	no	no	no	no	
154	no	no	yes	yes	no	no	no	no	no	no	yes	yes	no	no	no	no	yes	no	no	no	yes	no	yes	

* Victawet 58-B was too turbid to read in the 0.3 percent solution.

Table 6. The presence of growth of the Aerobacter organisms in a 0.3 per cent solution of each of the surface-active agents in the basic medium.

Culture number	Surface-active agents																						
	Aerosol OT	Alkanol B	Aresklene 400	Arctic Syntex-A Powder	Carbowax 1500 Di-Oleate	Drene	Duponol WA Paste	Duponol WA Flakes	Duponol LS Flakes	DuPont Retarder Lan	Igepal CA	Igepon AP	Igepon T	Lauryl Pyridinium Chloride	Polyethyl-Glycol 400 Monooleate	Santomerse 3	Span 80	Tergitol 4	Tergitol 7	Tergitol 8	Triton W-30	Triton X-400	Tween 80	Victawet 58-B*
10	yes	yes	no	yes	no	yes	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	yes	no	yes
15	yes	yes	no	yes	no	yes	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	yes	no	yes
18	yes	yes	no	yes	no	yes	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	yes	no	yes
19	yes	yes	no	yes	no	yes	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	yes	no	yes
47	yes	yes	no	yes	no	no	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	yes	no	yes
52	no	yes	yes	yes	no	yes	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	no	no	yes
55	no	yes	yes	yes	no	no	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	yes	no	yes
59	no	yes	yes	yes	no	no	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	yes	no	no
66	no	yes	yes	yes	no	yes	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	yes	no	yes
68	no	yes	no	yes	no	no	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	yes	no	no
70	no	yes	yes	yes	no	yes	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	no	no	yes
76	no	yes	no	yes	no	no	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	no	no	yes
80	yes	yes	no	yes	no	yes	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	yes	no	yes
82	yes	yes	no	yes	no	yes	no	no	no	no	no	yes	yes	no	yes	no	no	no	no	no	yes	no	yes
85	yes	yes	yes	yes	no	no	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	no	no	yes
91	yes	yes	yes	yes	no	yes	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	no	no	yes
93	no	yes	yes	yes	no	yes	no	no	no	no	no	yes	no	no	yes	no	no	no	no	no	no	no	yes
96	no	yes	yes	yes	no	no	no	no	no	no	no	yes	no	no	no	no	no	no	no	no	yes	no	yes
104	yes	yes	no	yes	no	no	no	no	no	no	no	no	yes	no	no	no	no	no	no	no	no	no	yes
109	no	yes	yes	yes	no	yes	no	no	no	no	no	yes	yes	no	yes	no	no	no	no	no	no	no	yes
140	yes	yes	no	yes	no	no	no	no	no	no	no	yes	no	no	no	no	yes	no	no	no	no	no	yes
145	yes	yes	no	yes	no	yes	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	yes	no	yes

* Victawet 58-B was too turbid to read in the 0.3 percent solution.

EXPERIMENT III

Selection of a Specific Surface-Active Agent as a Source of Carbon

Part 1

Further "Screening" of Surface-Active Agents

After finding which surface-active agent could be used as a source of carbon, it was thought that by varying the amount of the surface-active agent more definite results might be obtained. If it were possible to find a particular dilution of one of the surface-active agents that only the genus Escherichia could utilize as a source of carbon, the first goal of this research would be fulfilled. The results of Experiment II were reviewed and the surface-active agents that most nearly fulfilled the requirements were used in further dilutions. Aerosol OT, Drene, and Span 80 seemed to have the greatest possibilities.

Media. The same basic medium as used in Experiment II was used in this experiment. Dilutions of 0.1, 0.2, 0.3, and 0.5 percent of each of these three agents, Aerosol OT, Drene, and Span 80 were prepared and added to the basic medium of Experiment II.

Procedure. The basic medium was made up and the surface-active agents added. Five milliliter amounts of the surface-active agent medium adjusted to pH 7.0 were prepared in tubes and were sterilized by autoclaving at 121°C. for 15 minutes. Standard nutrient broth cultures were prepared and incubated for 24 hours at 37°C. for each organism and then used as the inoculum. The tubes of the surface-active agent medium were inoculated with

one loopful of the broth cultures of the organisms, the loop being a 20 gauge Nichrome wire loop with a 4 mm diameter. All cultures were incubated for 48 hours at 37°C. Growth during this period of time was determined by noting any visible turbidity. Any amount of growth was considered a positive test.

Results. The results of tests with the two genera are divided in order to facilitate interpretation, the surface-active agents being listed alphabetically. The results of the Escherichia species being subjected to the different surface-active agents are listed in Table 7. The results of the Aerobacter species being subjected to the different solutions of the surface-active agents are expressed in Table 8.

Table 7. The presence of growth of the Escherichia organisms in different dilutions of each of the surface-active agents in the basic medium.

Culture number	Surface-active agents											
	Aerosol OT, percent				Drene, percent				Span 80, percent			
	0.1	0.2	0.3	0.5	0.1	0.2	0.3	0.5	0.1	0.2	0.3	0.5
1	no	yes	yes	no	yes	yes	yes	yes	yes	yes	yes	yes
2	yes	yes	no	no	yes	yes	yes	yes	yes	yes	yes	yes
3	yes	yes	no	no	yes	yes	yes	yes	yes	yes	yes	yes
4	yes	yes	no	no	yes	yes	no	yes	yes	yes	yes	yes
5	yes	yes	no	no	yes	yes	yes	yes	yes	yes	yes	yes
6	yes	yes	no	no	yes	yes	yes	yes	yes	yes	yes	yes
7	yes	yes	no	no	yes	yes	yes	yes	yes	yes	yes	yes
8	yes	yes	no	no	yes	yes	yes	yes	yes	yes	yes	yes
13	no	no	no	no	yes	yes	no	no	no	no	yes	no
84	yes	yes	no	yes	yes	yes	yes	no	yes	yes	yes	no
87	yes	yes	no	no	yes	yes	yes	yes	yes	yes	yes	no
92	yes	yes	no	no	yes	yes	yes	yes	yes	yes	no	yes
97	yes	yes	no	no	yes	yes	no	no	no	no	yes	no
141	yes	no	yes	yes	yes	yes	no	no	yes	no	yes	no
154	yes	yes	no	no	yes	yes	no	no	no	no	yes	yes

Table 8. The presence of growth of the Aerobacter organisms in different dilutions of each of the surface-active agents in the basic medium.

Culture number	Surface-active agents											
	Aerosol OT, percent				Drene, percent				Span 80, percent			
	0.1	0.2	0.3	0.5	0.1	0.2	0.3	0.5	0.1	0.2	0.3	0.5
10	yes	yes	yes	yes	yes	yes	yes	yes	no	no	no	yes
15	yes	yes	yes	yes	yes	yes	yes	yes	no	no	no	yes
18	yes	yes	yes	yes	yes	yes	yes	yes	no	no	no	yes
19	yes	yes	yes	yes	yes	yes	yes	yes	no	no	no	yes
47	no	no	yes	no	no	no	no	no	no	no	no	no
52	yes	yes	no	no	yes	yes	yes	yes	no	no	no	no
55	no	no	no	no	yes	no	no	yes	no	no	no	no
59	no	no	no	no	yes	yes	no	yes	no	no	no	no
66	no	no	no	no	yes	yes	yes	yes	no	no	no	no
68	no	no	no	yes	yes	yes	no	no	no	no	no	no
70	no	no	no	no	yes	yes	yes	yes	no	no	no	no
76	no	no	no	no	yes	yes	no	yes	no	no	no	no
80	no	no	yes	no	yes	yes	yes	yes	no	no	no	no
82	no	no	yes	no	yes	yes	yes	yes	no	no	no	no
85	no	no	yes	no	yes	yes	no	yes	no	no	no	no
91	yes	yes	yes	no	yes	no	yes	yes	no	no	no	no
93	no	no	no	no	yes	no	yes	yes	yes	yes	no	no
96	yes	no	no	no	yes	no	no	yes	yes	yes	no	yes
104	no	no	yes	no	yes	no	no	yes	no	no	no	no
109	no	no	no	yes	yes	yes	yes	yes	no	no	no	no
140	no	no	yes	no	yes	yes	no	yes	yes	yes	yes	yes
145	yes	no	yes	yes	yes	no	yes	yes	no	no	no	no

Part 2

Utilization of Surface-Active Agents by "Non-Coliform" Organisms

After carefully studying the results in Tables 7 and 8, the 0.3 percent solution of the surface-active agent Span 80 seemed to give nearest the desired results. A large majority of the Escherichia cultures and only one of the Aerobacter cultures were able to utilize Span 80 as a source of carbon.

Since organisms other than "coliform" bacteria are also present in water, it was deemed advisable to see if other genera of microorganisms could utilize Span 80 as a source of carbon. If other genera were able to utilize this surface-active agent as a source of carbon, the test would lose much of its contemplated value.

Organisms of several genera were secured and inoculated into the 0.3 percent Span 80 medium. Thirty-eight different species were tested.

Medium. The basic medium as used in Part 1 of Experiment III was used in this experiment. Span 80 was added to produce a 0.3 percent concentration in the basic salts medium. The Span 80 was the sole source of carbon in the medium.

Procedure. The basic medium with the Span 80 added was adjusted to pH 7.0 and tubed in five-milliliter amounts. The tubes were then sterilized by autoclaving at 121°C. for 15 minutes. Standard nutrient broth cultures of each organism were prepared and used as the inoculum. The tubes of the surface-active medium were inoculated with one loopful of the broth cultures of the

organisms, the loop being a 20 gauge Nichrome wire 4 millimeter in diameter. All cultures were incubated for 48 hours at 37°C. Any amount of growth during this period of time was considered a positive test. Growth was ascertained by noting any visible turbidity.

Results. The results of the test are recorded in Table 9. The table lists the genus and species of the various organisms and whether they were able to grow and utilize the surface-active agent as the source of carbon.

Table 9. Growth of different species of microorganisms in the proposed Span 80 medium.

Microorganism	Evidence of growth
Achromobacter liquifaciens	no
Achromobacter superficiale	no
Actinomyces sp.	no
Alcaligenes fecalis	no
Alcaligenes recti	yes
Bacillus cereus	yes
Bacillus laterosporus	yes
Bacillus megatherium	yes
Bacillus polymyxa	yes
Bacillus pumilus	yes
Bacillus sphaericus	no
Bacillus subtilus	yes
Bacillus subtilus	yes
Bacillus subtilus	yes
Erwinia sp.	yes
Erwinia sp.	yes
Microbacterium flavum	no
Micrococcus aurantiacus	no
Micrococcus epidermidis	no
Micrococcus luteus	no
Micrococcus pyogenes var. aureus	no
Proteus mirabilis	no
Proteus vulgaris	no
Pseudomonas convexa	yes
Pseudomonas incognita	yes
Pseudomonas jaegeri	yes
Pseudomonas sp.	yes
Pseudomonas tomato	yes
Salmonella hirschfeldii	no
Salmonella schottmuelleri	yes
Salmonella typhosa	yes
Sarcina flava	no
Serratia marcescens	yes
Shigella alkalescens	yes
Shigella paradysenteriae Flexner U	yes
Shigella paradysenteriae type Newcastle	yes
Shigella sonnei	no
Vibrio metschnikovii	no

Part 3

Span 80-Sodium Propionate Mixture as a Carbon Source for Escherichia Species

As the results of Part 2 of Experiment III did not make possible a clear cut distinction of "coliform" from "non-coliform" organisms, it was deemed advisable to modify the medium further. By adding another constituent it was hoped it would make the test more specific for the "coliform" organisms. Propionic acid was decided upon as the needed constituent. Because propionic acid was not available, propionates were used. Calcium propionate when added to the medium formed a flocculent precipitate. Sodium propionate when added to the medium formed only a small amount of flocculent material, thus it could be used as a constituent. When ammonium sulfate was substituted for sodium ammonium phosphate, the flocculent material did not form.

The same "coliform" organisms and the organisms as used in Part 2 of Experiment III were inoculated into this modified medium.

Medium. The basic medium as used in Part 1 of Experiment III, with the substitution of ammonium sulfate for the sodium ammonium phosphate, was used in this experiment. Sodium propionate was added to produce a 0.3 percent concentration in the basic salts medium. Span 80 was added to the basic medium in conjunction with the sodium propionate to produce a 0.01 percent concentration of the Span 80.

The modified medium had the following composition:

$MgSO_4$	0.2 gram
KH_2PO_4	1.0 gram
Sodium Propionate	3.0 gram
Span 80	0.1 gram
$(NH_4)_2SO_4$	1.5 gram
Water	1000.0 milliliters

The basic medium with the Span 80 and sodium propionate was adjusted to pH 7.0 and tubed in five-milliliter amounts. The tubes were then sterilized by autoclaving at 121°C. for 15 minutes.

Procedure. The inocula for this experiment were made by suspending the growth from 24-hour agar slants of the organisms in sterile distilled water. The tubes of the medium were then inoculated with one 4 millimeter 20 gauge Nichrome wire loopful of the suspension of the organisms. All cultures were incubated for 48 hours at 37°C. Any amount of growth during this period of time was considered as a positive test. Growth was ascertained by noting any visible turbidity.

Results. The results of growth of the "coliform" organisms in the modified medium are presented in Table 10. The table lists the numbers of the organisms and whether they used the medium as a source of carbon.

The results of the various genera other than "coliform" types are presented in Table 11. The table lists the genus and species of the various organisms and whether they were able to use the medium as a source of carbon.

Table 10. The growth of the "coliform" organisms in the proposed Span 80-propionate medium.

Culture number	Microorganisms	Evidence of Growth
1	Esch. acidilactici	yes
2	Esch. coli	yes
3	Esch. coli	yes
4	Esch. acidilactici	yes
5	Esch. coli	yes
6	Esch. coli	yes
7	Esch. coli	yes
8	Esch. coli	yes
13	Esch. acidilactici	yes
84	Esch. freundii	yes
87	Esch. freundii	yes
92	Esch. coli	yes
97	Esch. intermedium	weakly positive
141	Esch. coli	yes
154	Esch. intermedium	weakly positive
10	Aero. aerogenes	no
15	Aero. aerogenes	no
18	Aero. aerogenes	no
19	Aero. aerogenes	no
47	Aero. cloacae	no
52	Aero. aerogenes	no
55	Aero. cloacae	no
59	Aero. aerogenes	no
66	Aero. aerogenes	no
68	Aero. cloacae	no
70	Aero. cloacae	no
76	Aero. aerogenes	no
80	Aero. aerogenes	no
82	Aero. aerogenes	no
85	Aero. cloacae	no
91	Aero. aerogenes	no
93	Aero. aerogenes	no
96	Aero. aerogenes	no
104	Aero. aerogenes	no
109	Aero. cloacae	no
140	Aero. aerogenes	no
145	Aero. aerogenes	no

Table 11. Growth of different species of microorganisms in the proposed Span 80-propionate medium.

Culture number	Microorganism	Evidence of growth
121	Achromobacter liquifaciens	no
122	Achromobacter superficiale	no
119	Actinomyces sp.	no
15	Alcaligenes fecalis	no
127	Alcaligenes recti	no
101	Bacillus cereus	no
94	Bacillus laterosporus	no
56	Bacillus megatherium	no
114	Bacillus polymyxa	no
5	Bacillus pumilus	no
103	Bacillus sphaericus	no
35	Bacillus subtilus	no
61	Bacillus subtilus	no
65	Bacillus subtilus	no
16	Erwinia sp.	no
152	Erwinia sp.	no
100	Microbacterium flavum	no
110	Micrococcus aurantiacus	no
20	Micrococcus epidermidis	no
147	Micrococcus luteus	no
2	Micrococcus pyogenes var. aureus	no
106	Proteus mirabilis	no
9	Proteus vulgaris	no
6	Pseudomonas sp.	no
89	Pseudomonas convexa	no
133	Pseudomonas incognita	no
132	Pseudomonas jaegeri	no
117	Pseudomonas tomato	no
2	Salmonella hirschfeldii	no
7	Salmonella schottmuelleri	no
168	Salmonella typhosa	no
77	Sarcina flava	no
3	Serratia marcescens	no
161	Shigella alkalescens	no
160	Shigella paradysenteriae, Newcastle	no
162	Shigella paradysenteriae Flexner U	no
21	Shigella sonnei	no
167	Vibrio metschnikovii	no

CONCLUSIONS

The main objective of this work was an attempt to modify or revise the test for the bacteriological examination of water. At present the "coliform" bacteria are used as indicators of pollution in the examination of water. The genus Escherichia, when present, indicates the possibility of pathogenic organisms being present. The genus Aerobacter is also included in the "coliform" group, but when present alone in water indicates the probability of only soil contamination. The differentiation between the two genera is not prescribed in the routine examination of waters at present. If it were possible to devise a simple test that would easily differentiate between these two genera, it would greatly enhance the methods for examination of water for "fecal" pollution.

Before using the various surface-active agents as possible sources of carbon, toxicity of the surface-active agents upon selected strains of "coliform" organisms had to be ascertained. The toxicity tests showed that the anionic and the non-ionic surface-active agents had little or no toxic effect on the "coliform" organisms, but the cationic agents for the most part were extremely toxic. Because of this fact the cationic surface-active agents could not be used in the experiments on utilization of surface-active agents as a source of carbon for cell metabolism.

The different surface-active agents were then added to a basic salts medium and checked to see if they could be used as a source of carbon by the genus Escherichia. Aerosol OT, Drene and Span 80 seemed to give the most promise as possible sources of carbon for

the genus Escherichia. These three surface-active agents were then further "screened" using different dilutions of each agent to see if some dilution other than a 0.3 percent level would give more uniform results. The 0.3 percent solution of the Span 80 seemed to give the closest to the desired results.

The thought that other organisms present in water might give false positive results motivated an experiment to subject organisms other than "coliform" bacilli to the Span 80 medium. Part 2 of Experiment III was devised to find out if it were possible for organisms other than the "coliform" group to utilize the Span 80 as a source of carbon. Genera other than the "coliform" group were able to utilize this medium, hence the applicability of the Span 80 medium was lowered by these findings.

Koser (1923) had found that propionic acid was utilized by the genus Escherichia and not by the genus Aerobacter. A medium was next devised in which sodium propionate was added as the source of carbon with the Span 80 being used to lower the surface tension and partially inhibit the genera other than the "coliform" group. The results of use of the Span 80-propionate medium were very successful inasmuch as the genus Escherichia was able to utilize the Span 80-sodium propionate medium as a source of carbon while none of the other genera tested was able to grow in this medium.

In review of the results of this work it is the opinion of the author that the bacteriological examination of water could be simplified. The following is a suggested revised procedure for the examination of water. Inoculate a lactose fermentation tube, such as is used at present, with the water sample. If after 48 hours'

incubation, gas is present in the fermentation tube, a tube of the Span 80-sodium propionate medium is inoculated from the fermentation tube. To prevent contaminating the proposed medium with carbonaceous matter from the fermentation tube, use a straight needle for inoculation. Since the tube of Span 80-sodium propionate medium will support growth of the genus Escherichia and will not support growth of other bacteria likely to be present any growth in the new confirmatory medium would be called positive for "fecal" pollution. By substituting the Span 80-sodium propionate medium for the confirmatory steps in the Standard Methods routine procedure, the time to complete the bacteriological examination of water would be greatly shortened.

Time has not permitted field tests of this modified procedure. Only evidence of the presence or absence of growth can be ascertained by use of the liquid medium. It is suggested that by the use of a solid medium of somewhat similar composition it might make possible the recognition of the colony formation of the genus Escherichia. If any other organisms should happen to grow accidently on the solid medium, the colony thus might be easily recognized. By this method the possible chance of reporting negative samples as positive (which could be caused by contamination) might be prevented.

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