A STUDY OF CHEMICAL AGENTS FOR SELECTIVE GROWTH OF THE COLIFORM ORGANISMS

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A STUDY OF CHEMICAL AGENTS FOR SELECTIVE GROWTH OF THE COLIFORM ORGANISMS
(An Abstract)

Since the first recognition that drinking water which had become contaminated with fecal material may be the source of dissemination of certain diseases, there has been a search for a suitable means of testing waters for bacterial purity.

Many selective media have been devised for the detection of the coliform organisms. Of this host of media, most have been abandoned because of inaccurate results obtained by their use. These inaccuracies were mainly due to too great a toxicity to the coliform organisms.

Even the confirmatory media most widely used today, brilliant green bile broth, and eosin methylene blue agar, have been shown by Mallmann and Darby[1] to be inhibitory to the coliforms. Thus false information is given by their use, and a false impression may be obtained regarding the condition of a water source.

As a result of these findings, it seemed advisable to continue the search for a selective agent which would effectively inhibit all non-coliform organisms which may give false positive tests. At the same time this agent must not in any way interfere with the growth or gas production by the members of the coliform group.

Seven substances were tested which were known to exhibit this selectivity to a greater or lesser extent.

Of these seven compounds, preliminary tests showed five to be inhibitory to growth and gas production by the coliforms at a concentration which would effectively inhibit the two gram positive organisms, Staphylococcus aureus and Bacillus subtilis.

These five compounds are:

1. Potassium ferrocyanide
2. Potassium ferricyanice
3. Potassium cyanate
4. Potassium arsenate
5. Potassium arsenite

The other two compounds tested were ethyl purple and 2,2' Methylene bis-4-chloro-6-isopropyl phenol (K-7643). Both of these compounds exhibited a remarkable selectivity in preliminary tests.

Ethyl purple effectively inhibited the gram positive organisms tested at a dilution as high as 1-1.1 million, while allowing the coliforms to grow at concentrations from 1-40,000 to 1-10,000. Further studies with ethyl purple showed that a concentration of 1-333,000 would effectively inhibit the gram positive organisms without noticeable inhibiting gas production by the coliforms.

Compound K-7643 was shown to inhibit growth of the gram positive bacteria tested in a dilution of more than 1-1 million, and allowed abundant growth of the coliforms at 1-10,000, the highest concentration tested. Further studies using 1-100,000 and 1-400,000 K-7643 showed it to exhibit no apparent inhibition of the coliforms, while effectively inhibiting growth of the gram positive bacteria tested.

Actual field tests run with media containing these compounds in proper concentration on chlorinated sewage effluent showed them to be comparable in their selective specificity. When compared with brilliant green bile broth it was evident that 1-333,000 ethyl purple and 1-100,000 K-7643 eliminated certain non coliform organisms which produced gas in, and discolored brilliant green bile medium. These two media also were capable of allowing the growth of certain coliforms which, due to their attenuated condition or natural sensitivity

were not able to initiate growth or produce gas in brilliant green bile medium.

The promising results in these limited studies of these two media seems to warrant their further and more intensive investigation as a possibly more accurate and dependable confirmatory medium for the detection of coliform organisms.

(1) Mallmann, W.L. and C.W. Darby. Uses of a Lauryl Sulfate Tryptose Broth for the Detection of Coliform Organisms., A.J.P.H. 31:127-134 (1941)

A STUDY OF CHEMICAL AGENTS FOR SELECTIVE GROWTH OF THE COLIFORM ORGANISMS

By

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In the last half century there has been a host of media devised for the selection and differentiation of organisms of the coliform group. The stimulus for the development of these many media was the recognition of a need for the detection of fecal pollution in water sources intended for domestic use, and later the need to test the efficiency of purification of known contaminated water sources being used for domestic consumption. The assumption and reasoning here being that in the case of fecal pollution, members of the coliform group will be present.

Theobald Smith (1) was the first to attempt the use of glucose broth in the estimation of Bacillus coli in water, He took advantage of the ability of B. coli to ferment glucose with the production of gas, and by inoculating tubes with various dilutions of the water sample being tested, he estimated the approximate numbers of organisms in the sample. It became evident however that many other organisms could also produce gas in glucose broth.

Jackson (2) in 1907 recommended the use of lactose broth containing bile for the elimination of non coliform gas producers. In 1912 lactose bile broth was the standard medium for the presumptive test in water analysis. Various workers, using different concentrations of bile broth reported conflicting opinions on the advisability of using bile as a presumptive medium. Some workers reported bile as being inhibitory to members of the coliform group, and as a result lactose broth without bile was recommended by the Standard Methods Committee in 1917.

Many species of bacteria occurring in water have the ability to ferment lactose while not belonging to the coliform group (3).

Among these species may be found members of the anaerobic spore forming genus Clostridium, namely Cl. perfringens and Cl. sporogenes. Also certain aerobic spore forming bacilli may produce gas from lactose. Many streptococci and some staphylococci have the ability to hydrolyze lactose into it's component monosaccharides glucose and galactose. These hexose sugars may then be attacked and broken down by certain bacteria such as some members of the genus Proteus to yield simpler products. Among these resultant products may be gasses. This synergistic combination may then result in false positive presumptive coliform tests. Various members of the genera Klebsiella and Erwinea may also ferment lactose with the production of gas, but their rare occurrence in water eliminates them from being significant as interfering organisms in water bacteriology.

It should be noted from the information just given that the great majority of false positive tests involve at least one gram positive species. Therefore, a desirable selective medium for use in water analysis should be one which would inhibit all gram positive organisms at a concentration which would not be inhibitory for the growth or gas production by the coliforms.

The interest in and development of various media used in water analysis proceeded in the following general manner.

Wurtz (4) in 1892 prepared lactose agar containing litmus as an indicator for the detection of intestinal lactose fermenting organisms. The litmus would turn red with acid production by the coliform organisms, and thereby indicate their presence.

Drigalski and Conradi (5) added gentian violet to the medium that Wurtz had devised. The gential violet being added to inhibit

the growth of gram positive organisms.

Endo (6) did his classical piece of work by using basic fuchsin decolorized with sodium sulfite for the isolation of Salmonella typhosa. Later various workers modified this medium for their particular purposes, and it became widely used for the detection and differentiation of the coliform organisms. The production of an aldehyde by E. coli on the medium restores the color to the basic fuchsin and produces a metallic sheen on the colony. The chief disadvantage of Endo's medium was it's instability. The color returns to the medium in a short time even at ice box temperatures in the dark, and much more rapidly under warm, light conditions.

The disadvantage of instability was overcome by Holt-Harris and Teague (7) who used eosin and methylene blue in their medium. This medium also underwent various modifications and simplifications. The modification by Levine (8) is still being used extensively today in the confirmatory test for coliforms in water.

Eisenberg (9) studied the action of 115 dyes on various microorganisms and he concluded that the gram positive organisms are generally much more sensitive to those dyes than are the gram negative organisms. Many other workers also recognized this fact and several dye media were devised which showed this specific selectivity to a greater or lesser extent.

Brief mention of some of these media devised for the isolation and/or detection of bacteria of the colon-typhoid group follows.

Conradi (10) in 1908 combined picric acid with brilliant green in a selective medium for the coliforms. Krumweide, Pratt and Mc-

Williams (11) also recommended brilliant green, while Teague and Clurman (12) favored eosin and brilliant green for use in the isolation of S. typhosa from stools.

Loeffler (13) used malachite green, and Ditter (14) used china blue malachite green agar. Glassner (15) employed china blue metachrome yellow agar. Many other workers used various combinations of these and similar selective dyes. Most of these dyes however proved to be inhibitory to members of the coliform group at the concentrations necessary for use.

Bile or it's salts were extensively employed also because of their marked selectivity. MacConkey (16) used bile salt agar with neutral red while Rector (17) used dried whole bile. It was found however that the concentrations necessary for the desired inhibition of gram positive organisms was somewhat inhibitory to the coliforms as well.

Dominik and Lauter (18) described a methylene blue-brom cresol purple combination to be used as a confirmatory medium for coliforms in water. Hall and Ellefson (19) used gentian violet in lactose broth and obtained better results than with lactose broth alone. In subsequent studies (20) these same investigators found that increasing amounts of dye gave fewer false positive presumptives, but at the same time tended to inhibit members of the coliform group.

A little prior to this work Dronffenbrenner, Schlesinger and Soletsky (21) recognized that rosolic acid inhibited gram positive organisms, but not the gram negative ones. He suggested it's possible use as a differential medium constituent.

Meur and Harris (22) showed a brilliant green bile medium to

have marked selective properties. This medium was extensively investigated and modified by various workers. (23,24) The final conclusion regarding this medium was that 2% brilliant green bile inhibited somewhat certain attenuated forms of the coliform bacteria, but at the same time it is probably slightly more efficient in detecting and differentiating the coliforms from other bacteria than is plain lactose broth.

Salter, (25) in his observations on the rate of growth of E. coli found that even at dilutions of one to one million or more, brilliant green and crystal violet inhibit the growth of E. coli. He also found that bile salts stimulate the growth of E. coli in a concentration of 0.5 percent. Advantage was taken of this finding by Hajna and Perry (26) in the formulation of their "E. C." medium.

Recently also other selective media have been investigated. Cowls (27) describes the use of Drene shampoo in a modified lactose broth as a possible improvement in the test for the coliform group.

Faulkner (28) showed that the female hormones diethylstilboestrol, hexoestrol, and stilboestrol are much more selectively inhibitory for the gram positive than for the gram negative organisms. He showed the coliforms to grow in concentrations of 1 to 5,000, while the gram positive organisms and some spore formers were killed in the range of 1 to 100,000 to 1 to 250,000.

Naghski, Copley, and Couch (29) showed quercetin to be inhibitory to certain gram positive organisms at concentrations of .075 to .1 milligrams per milliliter, the action being much less

on those of the gram negative group. The actual tolerance of these gram negative organisms was not determined however, since the practical limit of solubility of quercetin is about .15 milligrams per milliliter.

Recognition of the fact that many antibiotics show a remarkable selectivity quite naturally led to their investigation as a possible constituent of media to be used in the analysis of water.

Rittenberg and Silliker (30) tested three antibiotics against several organisms. They found that streptomycin had about the same effect on the coliform and non-coliform organisms, and eliminated it as of no possible value for the detection of coliforms.

In testing penicillin however they found that all coliforms tested could withstand 50 units per milliliter, while Cl. welchii and the enterococci were inhibited at 30 units. Five of the seven aerobacillus strains however were unaffected by concentrations up to 400 units per milliliter.

It was found in the case of tyrothricin that all 45 strains of coliforms tested withstood 100 micrograms per milliliter. All Cl. welchii and enterococci were inhibited by 30 micrograms per milliliter or less, while 4 out of the seven aerobacilli produced gas with as much as 50 micrograms per milliliter.

Actual field tests subsequently run proved antibiotics to possess no particular advantage over the presently used brilliant green bile broth. It was concluded by these investigators that at least for the present, the use of antibiotics in water analysis is not practical.

Chapman (31) discussed the remarkable selectivity of sodium

alkyl sulfate (called Tergitol 7). He claims that the only gram negative organisms inhibited by this substance are members of the genus Proteus.

Stark (32) in 1936 evaluated the use of formate ricinoleate broth for the detection of coliform organisms in milk. Later Frederiq and Levine (33) showed all cultures of E. coli tested as well as Aerobacter and Citrobacter species would produce acid and gas in formate ricinoleate medium.

Vaughn, Levine, and Smith (34) describe a buffered boric acid lactose medium for enrichment and identification of E. coli.

The 9th edition of Standard Methods (35) includes crystal violet lactose broth, formate ricinoleate broth, fuchsin lactose broth, and brilliant green bile broth as well as eosin methylene blue agar and Endo's agar as confirmatory media for positive lactose presumptive tubes in the test for coliform organisms in water.

Ruchhoft and Norton (36) found brilliant green bile, crystal violet, and formate ricinoleate broths to be less productive for coliforms than standard lactose broth.

Mallmann and Darby, (37) in their studies on the use of lauryl tryptose broth for the detection of the coliform organisms in water, found that eosin methylene blue agar and brilliant green bile broth cannot be used if it is desired to confirm all positive presumptive tubes which contain coliforms. Evidence to support their statement came from the fact that they were able, in many cases, to isolate coliform organisms from lauryl tryptose positive presumptive tubes which had failed to confirm by the use of eosin methylene blue agar or brilliant green bile broth.

The failure of these coliforms to confirm on eosin methylene blue or brilliant green bile indicates the probability that these media are too inhibitory to certain members of the coliform group such as might be found in marginal and insufficiently treated waters; that is, those waters which may contain coliforms in rather small numbers, or possibly coliforms somewhat attenuated by age, exposure to chlorination and other adverse conditions.

It is recognized that occasional outbreaks of water borne diseases occur even today from presumably tested and verified safe sources. This fact also may suggest that perhaps the waters involved in the etiology of such outbreaks are of this borderline type, and are missed by the routine media and methods of confirmation in use today.

A recognition of the above facts and indications then seems to deem it advisable to seek a confirmatory medium which will effectively inhibit the greatest proportion of non-coliform lactose fermenters without in any way inhibiting members of the coliform group.

The purpose of this work then is to survey a number of compounds with the hope of finding one which at the proper concentration will exhibit the desired degree of selectivity.

EXPERIMENTAL PROCEDURE:

The experiments conducted in this work were designed to satisfy a threefold objective.

1. (a) To determine the maximum concentration of chemical which would allow growth of the coliforms.

(b) To determine the minimum concentration of chemical which would effectively inhibit the growth of the gram positive organisms.

Due to low limits of solubility of some of the compounds tested, part (a) above could not be determined.

2. To determine the optimum concentration which would effectively inhibit the gram positive organisms without perceptibly interfering with gas production by the coliforms, if such an optimum concentration exists.

3. To determine the actual inhibitory action of concentrations of the chemicals within the range of dilutions between maximum tolerance by the coliforms, and minimum dose inhibitory for the gram positive organisms, as determined in (2) above. This was done by using minimal known inocula and plating known dilutions of the culture and determining growth curves over the initial stages of growth. A typical E. coli culture was used in these growth curve determinations.

The base medium used in all determinations throughout this work was one formulated by Darby and Hallmann (38). They showed that it's composition and pH provides optimum conditions for growth and gas production by the coliforms.

The composition of this base medium is:

Bacto tryptose----------2.0%

Bacto lactose----------0.5%

KH_2PO_4----------0.27%

K_2HPO_4----------0.27%

NaCl----------0.5%

The pH of this medium without adjustment is 6.8. This pH was shown by Darby and Mallmann to be optimum for the early growth phases of the coliforms.

The procedure for determining the maximum concentration of chemical tolerated by the various organisms tested involved the preparation of 10 milliliter portions of medium containing various dilutions of the chemical. These tubes, after sterilization in the autoclave at 121° C for 15 minutes, were seeded with a 4 millimeter loopful of a vigorously growing 24 hour culture of the organism being tested. Growth was determined after 24 hours by observing for visual turbidity of the medium. Incubation temperature was 35° C. in each case.

Once the approximate tolerance of the organisms to the chemical was obtained in the manner described above, fermentation tubes were prepared containing concentrations of the chemical embracing the range from maximum tolerance of the coliforms to minimum concentration inhibitory to the gram positives. These tubes then were also seeded with 4 millimeter loopfuls of 24 hour cultures of several strains of E. coli, A. aerogenes, and intermediate coliforms. Observation of amount of gas produced in these various concentrations, and comparison with gas production by the same organisms in the control tubes containing only base medium, establishes the highest concentration of the chemical which apparently would not inhibit gas production when observed at 12 and 24 hours.

The laboratory cultures of coliforms in this study will largely be referred to by number.

The following is a brief description of those cultures.

Culture number	Organism	Source	IMViC formula
1.	E. coli	water	+ + - -
2.	E. coli	water	+ + - -
3.	E. coli	man	+ + - -
4.	E. coli	cow rumen	+ + - -
5.	E. coli	cow feces	+ + - -
6.	E. coli	cow rumen	+ + - -
7.	E. coli	man	+ + - -
8.	E. coli	orange juice	+ + - -
9.	E. coli	water	+ + - -
10.	A. aerogenes		- - + +
11.	E. freundii		+ - + -
12.	E. coli	water	+ + - -
13.	E. coli	orange juice	+ + - -
14.	E. coli	man(atypical)	+ + - -
15.	E. coli	cow feces	+ + - -
16.	E. coli	water	+ + - -
17.	E. coli	water	+ + - -
18.	E. coli	water	+ + - -
24.	intermediate	water	+ + + -
26.	A. aerogenes	water	- - + +
27.	intermediate	water	+ + + -
28.	intermediate	water	+ - - +
30.	A. aerogenes	water	- - + +
32.	A. aerogenes	water	- - + +
33.	A. aerogenes	water	- - + +

The chemical compounds tested in this work are:

Potassium ferrocyanide	Baker's C.P.
Potassium ferricyanide	Eimer and Amend C.P.
Potassium cyanate	Baker's C.P.
Potassium arsenate	Baker's primary crystals C.P.
Potassium arsenite	Baker's C.P.
Ethyl Purple (Ethyl Violet) Hexaethyl triamino triphenyl methane	Harleco-Hartman Leddon Co. Philadelphia, Pa.

K-7643 (2,2 Methylene bis-4-chloro-isopropyl phenol) from Dow Chemical Co. Midland, Michigan.

The experimental results obtained with each of the above compounds and a discussion of these results will be taken up in order in the following section.

EXPERIMENTAL RESULTS OBTAINED WITH POTASSIUM FERROCYANIDE (Baker's C.P.)

Table I

Influence of Potassium Ferrocyanide on the Growth of Several Organisms. (Observed after 24 hours incubation at 35° C.)

	Concentration			
Culture	Control	1-25	1-50	1-100
10. A. aerogenes	+	+	+	+
11. E. freundii	+	+	+	+
12. E. coli	+	+-	+	+
B. subtilis	+	+ sl.	+	+
Staph. aureus	+	-	-	+

+ indicates visible growth.
- indicates no visible growth.
sl. slight growth.

The above table of results shows that Staph. aureus is inhibited by dilutions as high as 1-50, B. subtilis being more resistant, showing slight growth even at a concentration of 1-25.

The three coliforms tested here showed growth even at the 1-25 dilution. To be of any use for the selection of coliforms, potassium ferrocyanide must be used in a concentration higher than 1-25.

Table II

Influence of 1-25 Dilution of Potassium Ferrocyanide on Gas Production by Several Coliform Organisms in 24 Hours at 35° C.

Culture	Control	1-25
1. E. coli	70*	0 ≠
2. E. coli	75	0 ≠
4. E. coli	60	0 ≠
5. E. coli	70	0 ≠
17. E. coli	45	0 ≠
33. A. aerogenes	65	0 sl.
12. E. coli	55	5
B. subtilis	≠	≠ sl.
Staph. aureus	≠	-

≠ indicates growth.
- indicates no growth.
sl. indicates slight growth.
* denotes percent of gas produced in Durham fermentation tubes.

The above data indicate that potassium ferrocyanide at concentrations sufficient to effectively inhibit the growth of B. subtilis and Staph. aureus also is inhibitory to the production of gas by members of the coliform group. Consequently it would probably not be of use as a selective agent for the detection of the coliform group.

EXPERIMENTAL RESULTS WITH POTASSIUM FERRICYANIDE. (Eimer and Amend C.P.)

Table III

The Influence of Two Concentrations of Potassium Ferricyanide on the Growth of Several Organisms.

Culture	Control	Concentrations 1-50	1-100
10. A. aerogenes	≠	≠	≠
11. E. freundii	≠	≠	≠
12. E. coli	≠	≠	≠
B. subtilis	≠	≠	≠
Staph. aureus	≠	-	≠

≠ indicates growth.
- indicates no growth.

From the above table it is evident that concentrations in excess of 1-50 must be used to effectively inhibit B. subtilis. Therefore, to be of use as a selective agent for detection of coliforms, a concentration of more than 1-50 is necessary.

Table IV

Effect of 1-50 Potassium Ferricyanide on Gas Production by Various Coliform Organisms.

Culture	Control	1-50
1. E. coli	70*	0 sl.
2. E. coli	75	0 sl.
4. E. coli	60	0 sl.
5. E. coli	70	-
17. E. coli	45	-
33. A. aerogenes	65	0 sl.
B. subtilis	/	-
Staph. aureus	/	-

* indicates percent gas produced.
/ indicates growth.
- indicates no growth.
sl. indicates slight growth.

It may be concluded from the results obtained that potassium ferricyanide is inhibitory to growth and gas production by the members of the coliform group at concentrations necessary to effectively inhibit growth of the two gram positive organisms tested.

EXPERIMENTAL RESULTS OBTAINED WITH POTASSIUM CYANATE. (Baker's C.P.)

Table V

Influence of Potassium Cyanate on Growth of Several Organisms.

Culture	Control	1-25	1-50	1-100
10. A. aerogenes	≠	≠	≠	≠
11. E. freundii	≠	≠	≠	≠
12. E. coli	≠	≠	≠	≠
13. Serratia marcescens	≠	≠	≠	≠
14. B. subtilis	≠	-	≠	≠
15. Staph. aureus	≠	-	-	≠

The data in the table above show that concentrations of more than 1-50 are required to effectively inhibit B. subtilis, while Staph. aureus is inhibited by that concentration.

Table VI

Influence of 1-50 Potassium Cyanate on Gas Production by Several Coliform Strains.

Culture	Control	1-50
4. E. coli	60*	5
5. E. coli	70	0 ≠
6. E. coli	80	0 ≠
10. A. aerogenes	60	-
13. E. coli	70	0 ≠
15. E. coli	60	5
17. E. coli	70	7

* indicates percent of gas produced.
≠ indicates growth.
\- indicates no growth.

Since marked inhibition of gas production and growth by the coliforms is evident from the above data, it is unlikely that potassium cyanate would be of use in a selective medium for the detection of coliforms.

EXPERIMENTAL RESULTS OBTAINED WITH POTASSIUM ARSENATE.

(Baker's primary crystals C.P.)

Table VII

Influence of Two Dilutions of Potassium Arsenate on the Growth of Several Organisms.

	Concentration		
Culture	Control	1-50	1-100
10. A. aerogenes	≠	≠	≠
11. E. freundii	≠	≠	≠
12. E. coli	≠	≠	≠
B. subtilis	≠	-	≠
Staph. aureus	≠	-	≠

≠ indicates growth.
- indicates no growth.

The above table shows that potassium arsenate at 1-50 dilution inhibits growth of the two gram positive organisms tested, while at the same dilution the three coliforms were able to grow.

Table VIII

The Influence of 1-50 Potassium Arsenate on Gas Production by Several Members of the Coliform Group.

Culture	Control	1-50
1. E. coli	70*	25
2. E. coli	70	0 ≠
4. E. coli	60	-
5. E. coli	65	0 ≠
12. E. coli	55	5
17. E. coli	50	0 ≠
33. A. aerogenes	65	5
B. subtilis	≠	-
Staph. aureus	≠	-

* indicates percent of gas production.
≠ indicates growth.
\- indicates no growth.

It becomes obvious by examination of the above data that potassium arsenate in concentration necessary to inhibit the gram positive organisms tested also inhibits the gas production and growth by the coliforms. For that reason then it would not be satisfactory as a selective medium for the detection of the coliforms.

EXPERIMENTAL RESULTS OBTAINED WITH POTASSIUM ARSENITE

(Baker's C.P.)

Table IX

Effect of Various Concentrations of Potassium Arsenite on the Growth of Several Organisms.

Culture	Control	Concentration 1-2000	1-4000	1-20000
10. A. aerogenes	≠	≠	≠	≠
11. E. freundii	≠	≠	≠	≠
12. E. coli	≠	≠	≠	≠
B. subtilis	≠	-	≠ sl.	≠
Staph. aureus	≠	-	-	≠

≠ indicates growth.
- indicates no growth.
sl. indicates slight growth.

The above results indicate that concentrations in excess of 1-4000 are necessary to inhibit growth of Staph. aureus and B. subtilis.

Table X

Influence of Various Concentrations of Potassium Arsenite on the Production of Gas by Several Coliform Strains.

Culture	Control	Concentration 1-1000	1-2000	1-3000	1-4000
1. E. coli	30*	5	55	40	55
2. E. coli	75	0 ≠	0 ≠	0 ≠	0 ≠
4. E. coli	55	0 ≠	0 ≠	0 sl.	0 sl.
5. E. coli	40	0 ≠	0 ≠	0 ≠	7
17. E. coli	90	0 sl.	0 ≠	0 ≠	5
33. A. aerogenes	65	0 ≠	5	20	35
Ser. marcescens	≠	sl.	sl.	≠	≠
B. subtilis	≠	-	sl.	≠	≠
Staph. aureus	≠	-	sl.	sl.	sl.

* indicates percent of gas produced.
≠ indicates growth.
\- indicates no growth.
sl. indicates slight growth.

The above results show a varying degree of inhibition of growth and gas production of several coliforms at the concentration necessary to effectively inhibit growth of Staph. aureus and B. subtilis. For that reason it seems unlikely that potassium arsenite would be of use as a constituent in a medium selective for the coliforms.

EXPERIMENTAL RESULTS OBTAINED WITH ETHYL PURPLE. (Ethyl Violet)

The results shown in table XI agree with the findings of Darby (39). He also found that ethyl purple is inhibitory to the gram positive organisms at very high dilutions, and that members of the coliform group are able to grow in relatively high concentrations of the dye.

The data given here also show that E. coli is apparently more susceptible to the inhibitory properties of ethyl purple than is either A. aerogenes or E. freundii.

The next step in the investigation of the effects of ethyl purple on the coliform organisms is the determination of the influence of various concentrations of the dye on gas production by this group.

The method employed, involved the preparation of Durham fermentation tubes containing progressively greater concentrations of ethyl purple in the base medium. Inoculations were then made into these tubes using a 4 millimeter loopful of 24 hour cultures of coliforms obtained from various sources. Gas production was recorded as percent of gas in the fermentation inserts, readings being made at 12 and 24 hours and compared with control tubes similarly inoculated, but containing only the base medium.

It is evident from the results shown on table XII that ethyl purple in 1-80,000 concentration in some way inhibits the production of gas by coliform bacteria. This is especially apparent after 12 hours incubation, where indeed, 4 of the cultures even failed to show growth. The fact that many of those cultures which showed less gas than the controls at the 12 hour interval actually showed comparable percent of gas in 24 hours, indicates that the ethyl purple may act

Table XI

The Effect of Various Concentrations of Ethyl Purple on the Growth of Several Organisms.

Culture	Concentration							
	Control	1-10T	1-15T	1-20T	1-25T	1-30T	1-35T	1-40T
1. E. coli	≠	-	-	-	-	-	-	≠
12. E. coli	≠	-	-	-	-	≠	≠	≠
11. E. freundii	≠	-	≠	≠	≠	≠	≠	≠
10. A. aerogenes	≠	≠	≠	≠	≠	≠	≠	≠
26. A. aerogenes	≠	≠	≠	≠	≠	≠	≠	≠

Culture	Concentration						
	Control	1-1.1M	1-2M	1-3M	1-4M	1-5M	1-6M
B. subtilis	≠	-	≠	≠	≠	≠	≠
Staph. aureus	≠	-	-	-	-	-	-

≠ indicates growth.
- indicates no growth.

Table XII

The Effect of 1-80,000 Ethyl Purple on Gas Production by Several Coliform Strains.

	Control		1-80,000	
Culture	12 hrs.	24 hrs.	12 hrs.	24 hrs.
1. E. coli	60*	80	60	90
2. E. coli	20	60	5	50
3. E. coli	60	75	20	80
4. E. coli	60	60	10	40
5. E. coli	60	75	-	7
6. E. coli	50	75	✓	90
7. E. coli	80	90	10	60
8. E. coli	40	75	5	60
9. E. coli	60	75	7	80
11. E. freundii	20	85	20	80
13. E. coli	65	80	-	-
14. E. coli	45	60	45	75
15. E. coli	60	80	-	20
16. E. coli	60	80	40	90
17. E. coli	60	80	-	40
19. E. coli	30	50	7	75

* indicates percent of gas produced.
✓ indicates growth.
- indicates no growth.

by inhibiting or slowing down growth or division of the cells rather than actually supressing the gas forming mechanism proper.

Of the sixteen strains of coliforms grown in 1- 0,000 ethyl purple, seven strains showed rather marked inhibition. These seven strains were seeded into two higher dilutions of ethyl purple, 1-100,000 and 1-333,000. Gas production was recorded after 12 and 24 hours incubation at 35°C, as before.

Table XIII

The Effect of Two Concentrations of Ethyl Purple on Gas Production by Seven More Sensitive Coliform Strains.

	Control		1-100,000		1-333,000	
Culture	12 hrs.	24 hrs.	12 hrs.	24 hrs.	12 hrs.	24 hrs.
4. E. coli	20*	45	0 ✓	10	25	60
5. E. coli	25	55	5	55	20	50
6. E. coli	45	60	25	75	55	75
7. E. coli	55	80	25	55	60	85
13. E. coli	40	65	0	10	35	60
15. E. coli	50	80	5	55	40	60
17. E. coli	55	90	5	80	50	70

✓ indicates growth.
* indicates percent of gas produced.

By the results of the above table, it is shown that ethyl purple in a dilution of 1-100,000 still inhibits gas production by the coliforms to approximately the same extent as 1-80,000. However, at 1-333,000, gas production is comparable at both 12 and 24 hours to that produced in the corresponding control tubes.

Ethyl purple demonstrated a remarkable selective action against the gram positive bacteria tested. Also at the proper concentration, ethyl purple has practically no demonstrable toxicity to the coliforms, and for that reason it may be valuable as a selective agent in a medium for the detection of coliforms in water.

Revis (40) in 1911 in his work with malachite green, found that most coliform strains are inhibited by concentrations of the dye as low as .03 percent. He also found that on serial transplant in this dye, coliform strains lost all power to produce gas even when re-inoculated into ordinary media containing no dye. These strains however would show vigorous growth without producing gas. This production of permanently atypical coliforms may be due to an actual alteration of the cells metabolism by the dye, or by a selective process in which the non gas producers are favored.

Since ethyl purple (Hexaethyl triamino triphenyl methane) like malachite green, is a triphenyl dye, it was thought that possibly this same gas supressing phenomenon could be demonstrated by it's use. The proceedure employed in this experiment was much the same as that used by Revis. Two coliform strains (E. coli 1 and 2) were serially transferred each day in tubes of base medium containing 1-80,000 ethyl purple. This dilution had previously shown some inhibition to certain coliform strains, among which was E. coli 2. At the time of this writing these cultures have undergone 32 transfers, and still continue to produce abundant gas when re-transferred to base medium containing no dye.

EXPERIMENTAL RESULTS OBTAINED WITH 2,2'METHYLENEBIS-4-CHLORO-6-ISOPROPYLPHENOL (K-7643)

This substance was obtained by courtesy of the Dow Chemical Company of Midland, Michigan. The manufacturers state that K-7643 has a phenol coefficient against Staph. aureus of about 300, and against S. typhosa of from 1-10. They did not indicate how these coefficients were obtained, but it should be noted that K-7643 is not soluble in water to any appreciable extent under ordinary conditions.

In my attempts to render K-7643 soluble, several facts were observed. The substance is soluble in alcohol, glycerin, strong inorganic bases and distilled water when heated to boiling. However, none of the solutions obtained by these methods were satisfactory here, because as soon as the alcoholic solution was placed in an aqueous medium it precipitated out in the form of an amorphous, gummy substance. A similar reaction is obtained when the basic solution is brought near the neutral point, or when the hot solution cools to incubator temperature.

No true solution has thus far been obtained, but a rather stable, milky suspension was accomplished by the following means: 0.1 gram of solid K-7643 was dissolved in the smallest amount of ethyl alcohol necessary to render it soluble. Then 0.5 milliliters of Tween 80 (Atlas Products Company) was added and thoroughly shaken to produce an emulsion. This emulsion then was diluted to 100 milliliters, well shaken, stoppered and kept as a 1-1,000 stock emulsion.

Preliminary tests with this substance showed that K-7643 does exhibit considerable selective action against the gram positive organisms tested, while allowing the gram negative organisms to grow in the highest concentration used.

The Effect of Various Concentrations of K-7643 on the Growth of Several Organisms.

Table XIV

	Concentration				
Culture	Control	1-10T	1-100T	1-500T	1-1M
1. E. coli	≠	≠	≠	≠	≠
B. subtilis	≠	-	-	-	-
Staph.Aureus	≠	-	-	-	-

≠ indicates growth.
- indicates no growth.

Also at dilutions of 1-2M, 1-4M and 1-5M, the tubes did not become turbid with B. subtilis and Staph. aureus, but minute, granular growth showing bizarre forms under the microscope precipitated to the bottoms.

To determine the effect of two dilutions of K-7643 on gas production by various "typical"and intermediate coliform strains, fermentation tubes containing 1-100,000 and 1-400,000 K-7643 in the base medium were prepared and inoculated with a 4 millimeter loopful of the organisms being tested. Observations were made and recorded after 12, 24 and 36 hours incubation at 35° C.

From the results shown on Table XV, it appears that K-7643 in either dilution tested has no inhibitory effect on gas production by the various coliforms as compared with the amount of gas produced in the control tubes. Both these dilutions however did effectively inhibit the growth of both B. subtilis and Staph. aureus.

Further investigations on the influence of various concentrations of K-7643 on a strain of E. coli (No. 1) involved the more precise technique of seeding with minimal inocula, 100 milliliter portions of base medium containing the various concentrations of K-7643. After

0, 2, 4, and 6 hours incubation at 35° C., nutrient agar pour plates were seeded with measured volumes from the previously inoculated flasks of media. The plates were incubated for 24 hours at 35° C, and the colonies found to have developed were counted. From the known dilutions used for seeding the plates, the numbers of viable cells per milliliter were calculated for each concentration of K-7643, and for each time interval.

From the results obtained and recorded in Table XVI, it is evident that multiplication occurred at a comparable rate in all concentrations of K-7643 tested. This rate was also comparable to that of the control.

It may be concluded from this set of data that K-7643 shows no appreciable inhibition of growth of E. coli at the concentrations tested, and the previous data show it to be markedly inhibitory to the gram positive organisms tested with these and greater dilutions. Therefore K-7643 shows considerable promise and should be further investigated as a possible selective medium for coliform organisms.

Table XV

The Effect of Two Concentrations of K-7643 on Gas Production by Several Coliform Strains.

	Control			1-100,000			1-400,000		
Culture	12#	24	36	12	24	36	12	24	36
1. E. coli	5*	45	55	45	75	70	35	60	65
2. E. coli	20	50	60	7	70	70	10	45	70
7. E. coli	45	80	80	10	60	65	15	65	75
10. A. aerogenes	0	bub	bub	0	bub	bub	0	bub	bub
26. A. aerogenes	bub	bub	20	0	bub	20	0	bub	25
27. intermediate	bub	30	55	7	40	45	10	65	65
33. A. aerogenes	4	45	55	10	50	55	10	60	65
B. subtilis	✓	✓	✓	-	-	-	-	-	-
Staph. aureus	✓	✓	✓	-	-	-	-	-	-

* indicates percent of gas produced.
✓ indicates growth.
- indicates no growth.
bub indicates small bubble of gas.
# indicates incubation time in hours.

Table XVI

Comparison of Growth of E. coli (No. 1) in Various Concentrations of K-7643, with Growth in the Base Medium Alone.

	Incubation Period			
Concentration	0 hr.	2 hrs.	4 hrs.	6 hrs.
1-100T	33*	67	1950	138,400
1-400T	28	60	1855	140,400
1-600T	34	56	1816	138,400
1-1M	30	52	1949	107,900
Control	27	47	2317	113,700

* Number of organisms per milliliter.

Comparison of Results Obtained With 2 Percent Brilliant Green Bile, 1-333,000 Ethyl Purple, and 1-100,000 K-7643 on Chlorinated Sewage Effluent.

The following discussion involves information gained during the course of work concerned with determining the most efficient methods of effectively chlorinating sewage effluent at the Muskegon, Michigan Sewage Treatment Plant.

In this work lactose broth was used as the presumptive medium, and 2 percent brilliant green bile broth as the confirmatory medium for the detection of coliform organisms at various points in the treatment process.

It was noticed, especially in samples from chlorinated final effluent, that many lactose positive tubes failed to confirm in brilliant green bile broth. Microscopic examination of the contents of these tubes usually revealed the presence of gram variable to gram negative large spore-forming rods, with occasional gram positive cocci and also usually many gram negative, non sporing short rods.

Limited studies were made of 236 positive lactose tubes which failed to confirm on brilliant green bile broth. Tests using 1-100,000 K-7643, and 1-333,000 ethyl purple fermentation tubes respectively were run in parallel with brilliant green bile and observed in 24 and 48 hours. Of the 236 tubes which did not confirm with brilliant green bile, 18 confirmed with 1-100,000 K-7643, and 17 confirmed with 1-333,000 ethyl purple. It was possible to isolate, by streaking on solid nutrient agar, gram negative, non sporing, lactose fermenting, short rods from each of the tubes which had shown gas in either ethyl purple or K-7643 media. These data then indicate that of the 236 positive

lactose tubes which had failed to confirm on brilliant green bile, at least seven percent actually did contain coliforms. The brilliant green bile apparently was inhibitory to their development or their gas production.

It was further noticed that among the brilliant green bile positive tubes, there would frequently be produced a marked discoloration or yellowing of the medium. Two 4 millimeter loopfulls from 84 such discolored tubes were planted into tubes of ethyl purple and K-7643 broths, and the results obtained compared and presented in table XVII below.

Table XVII

Brilliant Green Bile Discolored	1-333,000 Ethyl Purple		1-100,000 K-7643	
*				
84+	65+	19-	63+	21-

* number of tubes.
\+ indicates presence of gas.
\- indicates no gas.

Microscopic examination of the contents of the brilliant green bile tubes which failed to produce gas when transferred to ethyl purple and K-7643 broths always revealed the presence of gram positive spore forming rods, often associated with shorter gram negative rods. It was thought that possibly the gram negative rods were coliforms responsible for gas production in the brilliant green bile, the gram positive spore formers merely being responsible for the discoloration.

To check this possibility, tryptose agar plates were streaked from those brilliant green bile tubes which did not produce gas in K-7643 or ethyl purple media. The colonies which developed were picked

and placed in lactose broth fermentation tubes. Although abundant growth took place in these tubes, in no case was gas produced in the inserts. These organisms were gram negative short rods usually, with occasional aberrant filamentous forms predominating. Failure to isolate the gram positive rods was probably due to the fact that streak plates were used, and since these gram positive rods did not develop, they were probably anaerobic forms. Lack of suitable anaerobic equipment made it impossible to isolate and further study these organisms.

It appears however that there are organisms, or a synergistic combination of organisms which may produce gas in brilliant green bile while not belonging to the coliform group.

Twenty of the discolored tubes which also produced gas in ethyl purple and K-7643 media were streaked on tryptose agar. Several colonies which developed were picked and seeded into lactose fermentation tubes. In every case it was possible to isolate gram negative, non sporing, lactose fermenting rods, although in three cases from K-7643, and in two cases from ethyl purple media, peculiar gram negative diptheroid forms were present, and apparently responsible for the gas production.

It is realized that no broad, general conclusions can be safely drawn from the limited observations made to date, but the information which is available from this single source indicates that 1-333,000 ethyl purple broth, and 1-100,000 K-7643 broth exhibit comparable selectivity for the coliform organisms, and appear to eliminate to as yet an unknown extent, false positive tests which are obtained with brilliant green bile broth. Also these two selective agents appear to allow the growth of certain coliforms which, due to their attenuated

or naturally sensitive condition cannot initiate growth or gas production in brilliant green bile.

Further studies with these selective agents should be undertaken in various localities, and under all practical conditions to determine if they actually do continue to show more accurate results than the presently used confirmatory media.

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