TRIS (HYDROXYMETHYL) AMINOMETHANE AND RELATED COMPOUNDS IN DILUTION WATERS AND THEIR EFFECT ON COLIFORM INDICES

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TRIS(HYDROXYMETHYL)AMINOMETHANE AND RELATED COMPOUNDS IN DILUTION WATERS AND THEIR EFFECT ON COLIFORM INDICES

BY

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AN ABSTRACT

Submitted to the College of Science and Arts
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The use of Tris(hydroxymethyl)aminomethane and several structural analogues in dilution waters was compared with phosphate buffer as to their effects on the coliform indices of river water samples.

Comparisons were made of the sample held in the dilution blanks for "zero-time" and twenty minutes. Tris exhibited increases over the phosphate buffer of 28%. for the "zero-time" and 59% for the twenty minute holding period when the tests were run at room temperature (21°C.).

There was no apparent benefit in using Tris rather than phosphate buffer as a diluent when the organism source was a pure culture or milk as compared to river water in which the coliforms are attenuated.

Tris is recommended as a replacement for Butterfield's phosphate buffer as a diluent in quantitative bacteriology.

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INTRODUCTION

Suitable bacteriological diluents are needed in all quantitative bacteriological work. Lack of suitable dilution waters may partially account for lower counts when the sample must first be diluted and then grown on artificial media than are usually obtained with direct microscopic counts. It became evident during the early 1920's that tap water and distilled water did not give consistent results and that better diluents would have to be found. Saline and various other inorganic salt solutions were experimented with during this decade. It was found that certain organisms could be diluted with physiological saline without any apparent undesirable effects, but for the majority of organisms some better diluent was needed.

With the work of Butterfield (1932) in the 1930's phosphate buffered water became a standard diluent and was adopted by many laboratories for routine dilution procedure. For many years saline and phosphate buffer were the two major dilution waters for vegetative cells, although plain distilled water was being used as the diluent for spore work. The use of tap water and most inorganic salt solutions was almost entirely discontinued during this period.

It became apparent that not even physiological saline or phosphate buffer were the optimum diluents for certain fastidious organisms and that a new type of dilution water would have to be found. Organisms such as Brucella and Vibrio seemed to decrease rapidly in any type of inorganic buffer, but survived well in complex organic solutions like dilute nutrient broth, dilute peptone broth, and other dilute protein solutions. In fact, Huddleson, et al (1960) were using a 0.05% tryptose saline solution as their standard Brucella diluent in the 1930's, recognizing that severe reduction in numbers occurred when physiological saline, phosphate buffer, or distilled water were employed as diluents.

In recent years these peptone or protein solutions have become popular with many workers in pathogenic bacteriology, but for people working with coliforms, staphylococci and other non-fastidious bacteria, phosphate buffer has remained the standard diluent. Individual workers have pointed out that phosphate buffers might be acting adversely on coliforms and that perhaps some type of an organic solution or buffer might be a better diluent.

Tris(hydroxymethyl)aminomethane (hereafter referred to as "Tris") had been known to exhibit buffering action in the pH range from 7 to 9, but no one had seriously suggested using it as a standard bacteriological diluent, even though it was being used in tissue culture work as a buffer of better than average properties.

Recent work showed that Tris gave increased counts of river coliforms as compared to phosphate buffer when using the membrane filter technique. It would seem that Tris might increase the coliform indices when using the multiple-tube dilution method, as well as increasing membrane filter counts. Working on this assumption, Tris, phosphate buffer, and a series of organic buffers were compared by the multiple-tube dilution method and by plate counts.

LITERATURE REVIEW

The search for suitable diluents has been sporadic since Butterfield proposed his phosphate buffer (0.0003M) in the early 1930's. In the decade or so preceeding Butterfield's work, there was considerable research done on inorganic salts as diluents. As early as 1917, sterile distilled water, sterile tap water, and sterile bottled water were investigated as dilution type waters by Rector and Daube (1917) and by Shearer (1917). Variability of results was common, although Shearer found that distilled water was much better than physiological saline (0.85% NaCl) as a diluent for meningococci..

The variability of results in tap and distilled water prompted a search for a more desirable diluent. Feeling that the pH changes due to microbial growth were detrimental to the bacteria, Cohen (1922) used phthalate and phosphate buffers to control these pH variances. His 0.05M phosphate buffer exhibited a slight toxicity, but was recommended because the variability of mortality became stabilized.

A series of studies on inorganic salts by Holm and Sherman (1921), Sherman and Holm (1922), Winslow and Falk (1923), Hotchkiss (1923) and Winslow and Brooke (1927) among others was undertaken

to determine the effect of numerous anions and cations on Escherichia coli. Sodium and potassium chlorides and phosphates appeared to be the most suitable inorganic salts for dilution work with E. coli. Much of the work was done to determine optimum salt concentrations for E. coli survival. Most of these workers used peptone solutions in combination with the various salt concentrations, but failed, except in case of Winslow and Brooke, to see any beneficial effect of these protein solutions. These workers suggested that perhaps the reduction in the lethal effects of these complex diluents might be due to the protective action of colloids, but no follow-up study suggested the use of peptone solutions as diluents.

Since distilled water, bicarbonate buffers, and saline were not the most suitable diluents for coliform work, Butterfield (1932) introduced his 0.0003M phosphate buffer, which is still the standard dilution buffer for many fields, including dairy and public health. Butterfield further demonstrated that some source water and Formula "C" buffer were as good or better than phosphate buffer in keeping counts from dropping off in the first half hour to hour. Source water made poor dilution water except in the case of Cincinnati tap water; and

since Formula "C" buffer was based on the phosphate buffer, he recommended the use of phosphate buffer.

In subsequent studies, Butterfield (1933) compared the various dilution waters by diluting the sample and then plating. He also found that the method of preparing bicarbonate buffer was extremely important. In the normal autoclaved bicarbonate dilution blank counts dropped rapidly; bicarbonate buffer sterilized by filtration in a Berkefeld-W-filter was less antagonistic to the bacteria; while if bicarbonate was aseptically added to autoclaved water the count did not diminish in the first 30 minutes. His source water dilution blanks were made by taking a sufficiently large enough sample so that one portion of it could be removed for autoclaving and then serve as the dilution water for the remainder of the sample. The phosphate buffer is made by adding 34 grams of potassium dihydrogenphosphate to 500 ml water, adjusting the pH to 7.2 with 1 N sodium hydroxide and diluting to one liter for stock solution. For buffer use, 1.25 ml of this phosphate stock is added per liter. Formula "C" buffer is phosphate buffer plus small amounts of calcium chloride (2.5 ml 0.1M), magnesium sulfate (2.5 ml 0.04M) and ferric chloride

(0.5 ml 0.001M) added to it. With slight concentration changes, this Formula "C" buffer is used today as dilution water for biological oxygen demand tests. Butterfield felt that there was a great advantage in having some minerals in the dilution water if at all possible.

Following the work of Butterfield, many workers experimented with various diluent solutions and their effects on coliforms. Saline solutions (0.85%) were found to be poor coliform diluents by Katzin, et al (1943) in their study of loss of lactose fermentation in coliforms isolated from dairy product sources. On the other hand, Igepon AP, an organic sodium sulfate compound manufactured by General Dyestuffs, was found to stimulate growth of coliforms in low concentrations. Baker, et al (1941) examined other organic substances also, but none were suggested as additions to standard coliform diluents.

Additional work with inorganic compounds was conducted by Bigger and Nelson (1941) after they found that talc powder, which had been accidentally rinsed from the inside of rubber tubing, supported coliform growth in distilled water. None of the various inorganic salts which were investigated received serious consideration as dilution waters, although some of them appeared to be as suitable as phosphate buffer for coliform dilution purposes.

Following the study of inorganic compounds, Nelson (1942) proceeded to use various organic materials in dilution waters. Many of them, such as ground-up acorns, linseed meal, powdered wood materials, etc., increased coliform counts, especially upon standing. Since none of these unusual, complex compounds were suggested for use as diluents, the only practical value of this research was the information that cork-stoppered bottles should be avoided for bacteriological work, since any amount of organic material in dilution bottles would support coliform growth.

Other cation effects on coliforms were studied by Tubiash (1952) and Heinmats, Taylor, and Lehman (1954). Bacteriostatic effect of nitrates on coliform growth was substantiated in work by Tubiash while investigating 'skip-patterns' (false negatives) in M.P.N. (most probable numbers) determinations of drinking water. In studies of heat and chemically treated E. coli where counts dropped from ten million to zero, the coliform counts remained at zero when the 'killed' suspensions were added to phosphate buffer or distilled water. On the other hand, the 'killed' coliforms did respond almost quantitatively to organic solutions containing any combination of Krebs cycle compounds. Heinmats

did not suggest, however, that any of these Krebs cycle compounds be used in dilution work.

In the last decade, workers began seriously suggesting that phosphate buffer might not be the best diluent for several organisms. Gunter (1954) found that Azotobacter and Pseudomonas did very poorly after being diluted in phosphate buffer. Garvie (1955) observed that E. coli survived as well in distilled water containing many dead organisms as it did in phosphate buffer. That Brucella abortus was rapidly destroyed in phosphate buffered saline, was determined by de Mello, et al (1951). These three investigations showed that organic solutions were the best diluents for any of these organisms.

Not only did Pacheco and Dias (1954) find that phosphate buffer was a poor bacterial diluent, but they proposed further that E. coli, Salmonella typhosa, and Staphylococcus aureus were lysed by phosphates in low concentrations. These Brazilians further discovered that phosphatolysis occurs with both living and dead bacteria. They found that washing the cells seemed to reduce lysis enormously and furthermore suggested that simple phosphate solutions activate the lytic principle which they called 'ferment'.

More currently, Straka and Stokes (1957) ran studies which showed the destructive action of Butterfield's phosphate buffer and distilled water on the bacteria of frozen poultry pies when compared to 0.5% peptone water by plate counts. The drop in count after 20 minutes and one hour standing in phosphate buffer was 12% and 37% respectively, distilled water 34% and 59% respectively, and peptone water 5% and 10% respectively. They found bacteria to be protected by proteinaceous substances. Counts increased in peptone water only after two hours of standing. The coliform organisms were less adversely affected by dilution in saline or distilled water than any other group of bacteria. Sugars in general were poor dilution agents at any concentration, while satisfactory dilution waters for frozen poultry pies included yeast extract, casein hydrolysate, neutralized glutamic acid, and glycine.

Among others Gomori (1946) showed phosphates and carbonates to be incompatible with calcium salts which might be found in water to be diluted. The ammonium salts were not stable enough, while barbitol proved to be slightly inhibitory to some enzyme systems and had extremely low solubility. Imidazole was good, compatible with calcium, but

too expensive. As mentioned by Gomori, three new, quite soluble, low-priced calcium compatible buffers were discovered. They were stable at 23° C. for at least three months They are: 2, 4, 6, collidine, a colorless liquid with a near neutral pH range; Tris(hydroxymethyl)aminomethane, colorless crystals with a slightly alkaline pH range; and 2 amino-2-methyl-1,3-propanediol, very much like Tris in structure and appearance but with a pH range closer to 8.0.

Tris is no newcomer to the buffer field, since it has been used for many years for buffering cell culture media. The standard bicarbonate buffer for tissue culture work was discontinued because it was difficult to control the pH, and it necessitated closed culture flasks in order to maintain the necessary carbon dioxide tension. Tris gave a fixed buffer and reduced the labor required for maintaining cultures. As reported by Swim and Parker (1955), the only apparent disadvantage of Tris seemed to be the appearance of intracellular granules in certain types of tissues.

Several workers have reported on other diluting agents which had noticeable effects on bacterial counts. In using versene (disodium versenate)

to keep iron from precipitating on the membrane filter, McCarthy (1956) found that versene increased coliform M.P.N.'s three to four times and increased coliform membrane filter counts about five times. Other than reducing surface tension, not much else is known about versene in this capacity.

Lipe and Mallmann (1960) found that surface reducing agent Triton X-100 (Rohn and Haas Co., Philadelphia) tended to break up Mycobacterium tuberculosis clumps in the concentration of 1-5,000. A representative set of results showed that in diluting a M. tuberculosis broth culture, the control showed 10.6 million organisms per ml, Tween-80 (surface reducing agent) about 16 million organisms per ml, and Triton X-100 (iso-octyl phenyl polethoxyethanol) nearly 27 million organisms per ml. After one day of standing, the respective counts were 50% lower (twice the clumping-no death), but the Triton X-100 counts were then still more than 2½ times higher than the control (no surface reducing agent in the diluent).

Other workers have also found that dispersion agents gave increased counts. In the correlation of bacterial growth with oxygen consumption, Greig and Hoogerheide (1941) needed dispersion agents. Using triethanolamine they found that increased

counts were the result of its dispersing activities. They felt that clumping takes place in any bacterial solution.

It is generally accepted that the type of diluent has no effect on spore survival. Recently Demain (1958) found that Bacillus spores did not decrease in distilled water while vegetative cells died fairly rapidly in tap water, distilled water, and physiological saline.

Green and Simmons (1960) in work with bacilli spores, found that irradiated spores died rapidly when held in phosphate buffer, saline, and distilled water, whereas the unirradiated spores did not decrease more than 10%. In further studies, these workers found that the irradiated spores had actually been changed to vegetative cells by the high energy rays. This would indicate that precautions must be taken against unsuitable diluents for work with irradiated spores.

MATERIALS

Buffers

In addition to comparing Tris with phosphate buffer, it was decided to compare structural analogues of Tris also. The other compounds chosen for the study were 2-hydroxymethyl-2-methyl-1,3-propanediol; 2-amino-2-methyl-1,3-propanediol; 2,2-dimethyl-1,3-propanediol; 2-nitro-2-methyl-1,3-propanediol; 2-nitro-2-hydroxymethyl-1,3-propanediol; and tertiary butyl alcohol. These chemicals were obtained from Eastman Organic Chemicals. Table 1 shows some of the important physical properties of each compound; Table 2 shows the structural formulas.

For specific information regarding ionization constants and heats of fusion of Tris and phosphate buffer the reader is referred to an article by Bernhard (1956).

Media

Lauryl Tryptose Broth was used as the presumptive medium. It is prepared from dehydrated Difco Bacto-Lauryl Tryptose Broth by dissolving 35.6 grams of the powder in 1000 ml of distilled water. The solution is distributed (7 ml) into 16 mm x 150 mm test tubes containing inverted fermentation inserts and sterilized by autoclaving for 15 minutes at 121°C. and 15 pounds pressure.

TABLE 1

PHYSICAL PROPERTIES OF COMPOUNDS TESTED

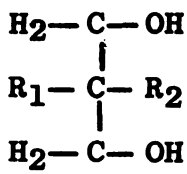
Diluent	Molec. Weight	Melt. Point	Boil. Point	Solubility in 100 ml Water
Tris	121.14	171°C	219°C	80 gm.
Hydroxymethyl-methyl	120.15	199	sublime	soluble
Amino-methyl	105.14	110	151	250
Dimethyl	104.15	127	203	soluble
Nitrohydroxy-methyl-methyl	151.12	165	decomp.	220
Nitro-methyl	135.12	148	decomp.	80
t-butanol	74.12	25	83	∞

Handbook of Chemistry and Physics, 36th Ed.

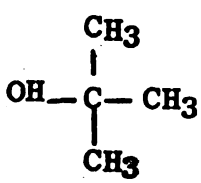
Table 2

THE STRUCTURAL FORMULAS OF THE VARIOUS COMPOUNDS

The basic structure as pictured on the right is 1,3-propanediol.



	R1 Substitute	R2 Substitute
1. Tris(hydroxymethyl) amino methane or 2-hydroxymethyl-2 amino-1,3-propanediol	$-CH_2OH$	$-NH_2$
2. 2-hydroxymethyl-2 methyl -1,3-propanediol	$-CH_2OH$	$-CH_3$
3. 2-amino-2-methyl-1,3-propanediol	$-NH_2$	$-CH_3$
4. 2,2-dimethyl-1,3-propanediol	$-CH_3$	$-CH_3$
5. 2-nitro-2-methyl-1 3-propanediol	$-NO_2$	$-CH_3$
6. 2-nitro-2-hydroxy-methyl-1,3-propanediol	$-NO_2$	$-CH_2OH$
7. tertiary butyl alcohol		



The constituents of Lauryl Tryptose Broth per liter are:

Bacto-Tryptose	20.0	g
Bacto-Lactose	5.0	g
Dipotassium Phosphate	2.75	g
Monopotassium Phosphate	2.75	g
Sodium Chloride	5.0	g
Sodium Lauryl Sulfate	0.1	g

The confirmatory medium is Difco's Bacto Brilliant Green Bile 2% (dehydrated). The broth is prepared by dissolving 40 grams of the powder in 1000 ml of distilled water and proceeding as above. The constituents of Brilliant Green Bile per liter are:

Bacto-Peptone	10.0	g
Bacto-Lactose	10.0	g
Bacto-Oxgall	20.0	g
Bacto-Brilliant Green	0.0133	g

The plate counts were made using Difco's Bacto-Plate Count Agar (dehydrated). The agar is made by dissolving 23.5 grams in 1000 ml of distilled water, distributing into flasks and autoclaving. The constituents of Plate Count Agar per liter are:

Bacto-Yeast Extract	2.5 g
Bacto-Tryptone	5.0 g
Bacto-Dextrose	1.0 g
Bacto-Agar	15.0 g

Equipment

Equipment included the Beckman pH meter (Model G) to determine the pH values of the organic buffers, the Cenco DuNouy Tensiometer to determine surface tensions, and the Quebec Colony Counter for making plate counts.

METHODS

Before testing the various organic compounds it was necessary to determine the optimum working concentrations for each. Preliminary work had proven that the optimum working concentration for Tris was 0.6%. Running ten sets of M.P.N.'s on seven different concentrations (0.01%, 0.05%, 0.1%, 0.4%, 0.7%, 1.0%, and 5.0%) of each compound, it was found that the optimum concentrations for tertiary butyl alcohol and hydroxymethyl-methyl propanediol was about 0.7%, for amino-methyl propanediol and dimethyl propanediol about 0.1%, and for nitro-methyl propanediol and nitro-hydroxymethyl propanediol about 0.01%.

Red Cedar River water was used as a source of coliform organisms and was suitably diluted before being added to lauryl tryptose broth fermentation tubes. The tubes were set up according to standard methods of water analysis using five tubes in each of three dilutions. This combination of fifteen tubes is called a set, and gives rise to one M.P.N. value from an M.P.N. index table, as set up in Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (1955). The various solutions of the seven compounds were used as diluents. Tris and the phosphate buffer controls

were made up in standard dilution blanks and sterilized by autoclaving for one half hour at 121°C. The other six organic compounds were made up as diluents by adding 27 ml of the appropriate concentration to extra large test tubes loosely plugged with No, 4 rubber stoppers and immersed in a 75°C. water bath for one half hour to kill any contaminating organisms since the confirmatory medium, brilliant green bile, would rule out any spore formers as false positives. In actual use, one-in-ten dilutions were made by adding 3 ml of water containing coliforms to this 27 ml dilution tube.

Each compound and phosphate buffer was tested as a diluent at two different time periods: one set of tubes was prepared immediately after the dilution had been made and the dilution blank had been shaken 35 times; the second set of tubes was prepared after the dilution blank or tube had stood on the counter at room temperature (21°C.) for twenty minutes and was shaken once sharply. Thus, each of the seven compounds was compared to phosphate buffer at "zero time" and after 20 minutes standing.

It is usually recommended that dilution blanks not be allowed to stand over twenty minutes in order to minimize the change in organism count, but it was felt that counts should be compared at both "zero-time"

and twenty minutes for all the diluent compounds including phosphate buffer in case a significant change occurred in any of them.

Sixty five sets of each compound at both time intervals were run and then compared to 65 sets of phosphate buffered water at both time intervals, with the exception that 85 sets of Tris at "zero-time" were compared to 85 sets of phosphate. The M.P.N. index value was converted to its corresponding logarithmic value for statistical analysis.

The degree of significance was determined by using the 't' distribution with 120 degrees of freedom. If the observed difference was significant at the level of 0.05 or less, it was then concluded that there is a real difference between the two diluents being compared.

Since Tris and t-butyl alcohol showed a significantly higher index than phosphate buffer using river water as a source of coliforms, the following research was done in order to determine whether similar results could be obtained using a pure culture of E. coli. The procedure followed for this research was identical to the preceding method, including the number of sets (65 of each) which were run. A twenty-four hour culture of E. coli which had been transferred daily for three

days before being used was suitably diluted so that it was only necessary to use three dilutions in each set in order to determine M.P.N. values.

One further comparison between Tris and phosphate buffer was made by the plate count method. A 24-hour suspension of E. coli was made up to contain ten thousand organisms per ml. This suspension was vigorously shaken fifty times. A two ml sample was withdrawn and one ml added to each of two dilution blanks, one containing 99 ml phosphate buffer, and one containing Tris buffer. Thus each of the two dilution blanks contained approximately one hundred organisms per ml at "zero time." Samples were withdrawn from both dilution blanks and plated immediately, 20 minutes, one hour, two hours, four hours, one day, and two days.

RESULTS

Table 3 shows a comparison of each compound by antilog of log average, median, and mode at zero minutes (length of time dilution blank stood after inoculation) and after standing at room temperature (21°C) for 20 minutes. All log averages were obtained by adding the logarithms of the individual M.P.N. values, dividing by the number of values, and converting the results into antilogs.

Table 4A shows a comparison of each compound at "zero-time" and after standing 20 minutes. Standard deviations were calculated and the per cent significance was determined by the 't' distribution. In interpreting the tables, the level of significance is determined solely from M.P.N. logarithmic averages. Medians, modes, per cent of M.P.N. values above average, and standard deviations only serve to substantiate the deductions which can be made from the M.P.N. logarithmic averages.

From Tables 3 and 4A, it can be seen that in most instances there is a good correlation between the logarithmic M.P.N. averages and the corresponding medians, modes, and per cent comparisons. The mode value for nitro-methyl propanediol is the only one which is out of line with other comparisons. There

TABLE 3

*CHANGE IN M.P.N. VALUES AFTER STANDING FOR 20 MINUTES IN EACH COMPOUND

Compound	Time of Exposure	M.P.N. Values Expressed As: Antilog of Log Ave.	Median	Mode
Phosphate	0	15,100	16,900	17,400
Phosphate	20	14,600	15,600	18,900
%Increase		-3.3	-7.7	8.6
Tris	0	18,500	22,000	24,000
Tris	20	21,800	22,000	24,000
%Increase		18	0.0	0.0
t-butanol	0	16,900	17,000	24,000
t-butanol	20	13,800	13,000	13,000
%Increase		-18	-24	-46
Hydroxymethyl-methyl	0	16,400	13,000	24,000
Hydroxymethyl-methyl	20	11,600	11,000	10,500
%Increase		-29	-15	-56
Amino-methyl	0	14,900	13,000	13,000
Amino-methyl	20	14,900	13,000	13,000
%Increase		0.0	0.0	0.0
Dimethyl	0	19,200	17,000	17,000
Dimethyl	20	17,600	22,000	12,500
%Increase		-8.3	23	-27
Nitrohydroxy-methyl-methyl	0	14,800	13,000	13,000
Nitrohydroxy-methyl-methyl	20	15,200	13,000	13,000
%Increase		2.7	0.0	0.0
Nitro-methyl	0	15,300	17,000	35,000
Nitro-methyl	20	14,100	13,000	13,000
%Increase		-7.8	-24	-63

*Source of sample-Red Cedar River.

TABLE 4A

*COMPARISON OF M.P.N. VALUES AT ZERO AND 20 MINUTES

Compound	Time of Exposure	Antilog of Log Ave.	%MPN's Above Average	%Signif. Zero Vs. 20 Min.
Phosphate	0	15,100	58.1	
Phosphate	20	14,600	51.8	60
Total Ave.		14,850		
Tris	0	18,500	53.8	
Tris	20	21,800	56.9	90
Total Ave.		20,110		
t-butanol	0	16,900	55.4	
t-butanol	20	13,800	49.2	95
Total Ave.		15,240		
Hydroxymethyl-methyl	0	16,400	46.2	
Hydroxymethyl-methyl	20	11,600	30.8	99.5
Total Ave.		13,740		
Amino-methyl	0	14,900	41.5	
Amino-methyl	20	14,900	44.6	<60
Total Ave.		14,900		
Dimethyl	0	19,200	44.6	
Dimethyl	20	17,600	52.3	76
Total Ave.		18,340		
Nitrohydroxy-methyl-methyl	0	14,800	43.1	
Nitrohydroxy-methyl-methyl	20	15,200	43.1	< 60
Total Ave.		15,020		
Nitro-methyl	0	15,300	52.3	
Nitro-methyl	20	14,100	41.5	71
Total Ave.		14,680		

*Source of sample-Red Cedar River.

were no significant (statistically) increases, but an appreciable increase did occur with Tris (18,500 to 21,800) after standing for 20 minutes.

A significant decrease occurred with both t-butyl alcohol (95% significance) and hydroxymethyl-methyl propanediol (99.5% significance). The respective decreases were 18% (16,900 to 13,800) and 29% (16,400 to 11,600). Phosphate buffer and the four remaining organic dilution waters were fairly constant, 8% being the greatest change made by any of them after standing for 20 minutes. It should be noticed that, except for one value of t-butyl alcohol, all M.P.N. indices fell within three standard deviations of the respective M.P.N. average, and that over 92% fell within two standard deviations of the average logarithmic value (see Table 4B).

Table 5A is a comparison of all compounds at both zero and 20 minutes holding time with phosphate buffer at "zero-time". All figures are adjusted to be directly compared against the phosphate values. Table 5B is a comparison of all compounds at both holding times with phosphate buffer at "zero-time". The per cent M.P.N.'s above average are adjusted to phosphate buffer at "zero-time" as being an even 50%.

Comparing all compounds at both holding times (including phosphate at 20 minutes) with phosphate

TABLE 4B

*COMPARISON OF M.P.N. VALUES AT ZERO AND 20 MINUTES

Compound	Time of Exposure	% of M.P.N. Indices Within: 1 Standard Deviation	2 Standard Deviations	3 Standard Deviations
Phosphate	0	73.8	95.9	100
Phosphate	20	62.6	95.4	100
Tris	0	74.2	96.5	100
Tris	20	70.8	92.3	100
t-butanol	0	56.9	96.9	98.5
t-butanol	20	67.7	95.4	100
Hydroxymethyl-methyl	0	75.4	95.4	100
Hydroxymethyl-methyl	20	72.3	95.4	100
Amino-methyl	0	83.1	98.3	100
Amino-methyl	20	63.1	92.3	100
Dimethyl	0	63.1	95.4	100
Dimethyl	20	69.2	95.4	100
Nitrohydroxy-methyl-methyl	0	69.2	95.4	100
Nitrohydroxy-methyl-methyl	20	66.2	98.5	100
Nitro-methyl	0	76.9	98.5	100
Nitro-methyl	20	69.2	96.9	100

*Source of sample-Red Cedar River.

TABLE 5A

*MPN VALUES OF EACH COMPOUND COMPARED TO PHOSPHATE (ZERO MINUTES)

Compound	Time of Exposure	M.P.N. Values Expressed As: Antilog of Log Ave.	Median	Mode
Phosphate	0	15,000	16,000	18,000
Phosphate	20	14,500	18,100	15,900
%Increase		-3.3	13	-12
Tris	0	19,200	21,300	25,400
%Increase		28	33	41
Tris	20	23,900	24,900	25,400
%Increase		59	56	41
t-butanol	0	19,500	20,900	33,200
%Increase		30	31	85
t-butanol	20	15,900	16,000	18,000
%Increase		6	0.0	0.0
Hydroxymethyl-methyl	0	18,900	16,000	33,200
%Increase		26	0.0	85
Hydroxymethyl-methyl	20	13,300	13,500	14,500
%Increase		-11	-16	-20
Amino-methyl	0	17,300	16,000	18,000
%Increase		15	0.0	0.0
Amino-methyl	20	17,200	16,000	18,000
%Increase		15	0.0	0.0
Dimethyl	0	16,100	12,400	12,800
%Increase		7.3	-23	-29
Dimethyl	20	14,800	16,000	9,400
%Increase		-1.3	0.0	-48
Nitrohydroxy-methyl-methyl	0	12,400	9,500	9,800
%Increase		-17	-41	-46
Nitrohydroxy-methyl-methyl	20	12,800	9,500	9,800
%Increase		-15	-41	-46
Nitro-methyl	0	12,800	12,400	26,300
%Increase		-14	-23	-46
Nitro-methyl	20	11,800	9,500	9,800
%Increase		-21	-41	-46

*Source of sample-Red Cedar River.

TABLE 5B

*M.P.N. VALUES OF EACH COMPOUND COMPARED TO PHOSPHATE (ZERO MINUTES)

Compound	Time of Exposure	Antilog of Log Ave.	Ave. of Each Cpd. & Phos. Zero Min.	% of MPN Indices Above Ave.	% Signif. Each Cpd. Vs. Phos. Zero Min.
Phosphate	0	15,000		50.0	
Phosphate	20	14,500	14,750	46.8	60
Tris	0	19,200	16,970	69.2	98
Tris	20	23,900	18,930	73.9	99.5
t-butanol	0	19,500	17,100	71.9	98
t-butanol	20	15,900	15,440	53.6	68
Hydroxymethyl-methyl	0	18,900	16,840	56.0	96
Hydroxymethyl-methyl	20	13,300	14,120	39.0	88
Amino-methyl	0	17,300	16,110	64.1	90
Amino-methyl	20	17,200	16,060	71.3	89
Dimethyl	0	16,100	15,540	43.9	71
Dimethyl	20	14,800	14,900	51.5	60
Nitrohydroxy-methyl-methyl	0	12,400	13,640	33.3	93
Nitrohydroxy-methyl-methyl	20	12,800	13,860	33.0	90
Nitro-methyl	0	12,800	13,860	40.5	87
Nitro-methyl	20	11,800	13,300	31.8	97

*Source of sample-Red Cedar River.

at "zero-time", several diluting compounds showed a significant increase (Tables 5A and 5B). These were: Tris at "zero-time" (98% significance), Tris at 20 minutes (99.5% significance), t-butyl alcohol at "zero-time" (98% significance), and hydroxymethyl-methyl propanediol at "zero-time" (96% significance). Their respective increases were 28% (15,000 to 19,200), 59% (15,000 to 23,900), 30% (15,000 to 19,500), and 26% (15,000 to 18,900). Nitro-methyl propanediol showed a significant decrease (97% significance) in 20 minutes, 21% drop (15,000 to 11,800). The remaining diluents varied from a 17% decrease to a 15% increase, none of which are statistically significant. As before, most of the medians, modes, and per cent changes substantiate the M.P.N. logarithmic averages.

Table 6A is a comparison of all compounds at both holding times with phosphate buffer at 20 minutes. All values are adjusted to be compared directly against the phosphate 20-minute values. Table 6B is similar to Table 6A except the data are treated by different means. The figures are adjusted for direct comparisons.

On the comparison of all compounds at both dilution exposure times (including phosphate at "zero-time") with phosphate buffer at 20 minutes

TABLE 6A

*MPN VALUES OF EACH COMPOUND COMPARED TO PHOSPHATE (20 MINUTES)

Compound	Time of Exposure	M.P.N. Values Expressed As: Antilog of Log Ave.	Median	Mode
Phosphate	20	15,000	18,600	16,400
Phosphate	0	15,500	16,400	18,600
%Increase		3.3	-12	13
Tris	0	19,900	21,900	26,200
%Increase		33	18	60
Tris	20	24,700	25,600	26,200
%Increase		65	38	60
t-butanol	0	20,200	21,500	34,200
%Increase		35	16	109
t-butanol	20	16,400	16,400	18,600
%Increase		9.3	-12	13
Hydroxymethyl-methyl	0	19,600	16,400	34,200
%Increase		31	-12	109
Hydroxymethyl-methyl	20	13,800	13,900	15,000
%Increase		-8	-25	13
Amino-methyl	0	17,900	16,400	18,600
%Increase		19	-12	13
Amino-methyl	20	17,800	16,400	18,600
%Increase		19	-12	13
Dimethyl	0	16,700	12,700	13,200
%Increase		11	-32	-20
Dimethyl	20	15,300	16,400	9,700
%Increase		2	-12	-41
Nitrohydroxy-methyl-methyl	0	12,800	9,800	10,100
%Increase		-15	-47	-38
Nitrohydroxy-methyl-methyl	20	13,200	9,800	10,100
%Increase		-12	-47	-38
Nitro-methyl	0	13,200	12,700	27,100
%Increase		-12	-32	+65
Nitro-methyl	20	12,200	9,800	10,100
%Increase		-19	-47	-38

*Source of sample-Red Cedar River.

TABLE 6B

*M.P.N. VALUES OF EACH COMPOUND COMPARED TO PHOSPHATE (20 MINUTES)

Compound	Time of Exposure	Antilog of Log Ave.	Ave. of Each Cpd. & Phos. Zero Min.	% of MPN Indices Above Average	% Signif. Each Cpd. Vs. Phos. Zero Min.
Phosphate	20	15,000		50.0	
Phosphate	0	15,500	15,250	53.4	60
Tris	0	19,900	17,280	73.9	99.5
Tris	20	24,700	19,250	78.9	99.5
t-butanol	0	20,200	17,410	76.8	99.5
t-butanol	20	16,400	15,680	57.3	92
Hydroxymethyl-methyl	0	19,600	17,150	59.8	99.5
Hydroxymethyl-methyl	20	13,800	14,390	41.7	60
Amino-methyl	0	17,900	16,390	68.5	99
Amino-methyl	20	17,800	16,340	76.2	98
Dimethyl	0	16,700	15,830	46.9	70
Dimethyl	20	15,300	15,150	55.0	60
Nitrohydroxy-methyl-methyl	0	12,800	13,860	35.6	91
Nitrohydroxy-methyl-methyl	20	13,200	14,070	35.3	89
Nitro-methyl	0	13,200	14,070	43.3	87
Nitro-methyl	20	12,200	13,530	33.9	95

*Source of sample-Red Cedar River.

(Tables 6A and 6B), the following were significantly higher statistically: Tris at "zero-time" (99.5% significance), Tris at 20 minutes (99.5% significance), t-butyl alcohol at "zero-time" (99.5% significance), hydroxymethyl-methyl propanediol at "zero-time" (99.5% significance), amino-methyl propanediol at "zero-time" (99% significance), and amino-methyl propanediol at 20 minutes (98% significance). The per cent increases can be read directly from Table 6A.

Nitro-methyl propanediol appeared to be significantly lower again (95% significance). The remaining dilution agents did not change significantly, but ranged from 15% lower to 11% higher than phosphate after 20-minutes standing. Also, as before, there was very good comparison between the logarithmic averages and the medians, modes, and per cent changes.

Tables 7 and 8 are a comparison of phosphate buffer, Tris, and t-butyl alcohol by the multiple tube dilution method (M.P.N.) using a pure culture of E. coli. The culture was transferred successively for three days before being diluted and used as an organism source. These tables (7 and 8) show that there is not much difference among the various component sets except in the mode values. This variability in mode values is probably due to an

TABLE 7

COMPARISON OF M.P.N. VALUES OF PHOSPHATE, TRIS, AND t-BUTYL ALCOHOL WITH PURE CULTURES AND HOLDING FOR ZERO MINUTES

Compound	M.P.N. Values Expressed As: Log Ave.	Median	Mode	% of MPN's Above Ave. of Cpd. & Phos.	% Signif. Each Cpd. Vs. Phos.
Phosphate	11,220	13,000	17,000	50.0	-
Tris	12,120	14,000	54,000	51.5	67
t-butanol	10,660	11,000	11,000	42.9	61
% Increase of Tris Over Phos.	8.1	7.7	218		
% Increase of t-butanol Over Phos.	-5.0	-15.0	-35		

TABLE 8

CHANGE IN M.P.N. VALUES AFTER STORAGE FOR 20 MINUTES IN EACH COMPOUND USING PURE CULTURES

Compound	Time of Exposure	M.P.N. Values Expressed As: Log Ave.	Median	Mode	% Signif. of Second to First Cpd.
Phosphate	0	11,220	13,000	17,000	
Phosphate	20	11,700	13,000	24,000	60
%Increase		4.3	0.0	41	
Tris	0	12,120	14,000	54,000	
Tris	20	12,610	13,000	7,900	58
%Increase		4.0	-7.1	-85	
t-butanol	0	10,660	11,000	11,000	
t-butanol	20	9,500	11,000	4,900	72
%Increase		-11	0.0	-55	
Phosphate	20	11,700	13,000	24,000	
Tris	20	12,610	13,000	7,900	65
%Increase		7.8	0.0	-67	
Phosphate	20	11,700	13,000	24,000	
t-butanol	20	9,500	11,000	4,900	87
%Increase		-19	-15	-80	

insufficient number of samples. There are no significant changes; most value differences are easily within experimental error.

Table 9 is a comparison of results using plate counts of pure E. coli cultures to compare effects of standing in phosphate buffer and Tris dilution waters. The holding times were: zero minutes, 20 minutes, one hour, two hours, four hours, and eight hours; data from one and two days holding periods at 21°C are also included.

Table 10 is an expansion of two sets of comparisons from Table 9--phosphate (zero minutes) versus Tris (four hours) and phosphate (zero minutes) versus Tris (eight hours). Significant differences in both comparisons are apparent (the reason for making an expanded comparison of the data in these two cases).

A comparatively short study was made between phosphate buffer and Tris buffer using pasteurized homogenized milk for an organism source. The results in Table 11 show similar colony counts with Tris and phosphate buffers.

In order to examine the dispersion possibilities, the surface tensions of Tris, t-butyl alcohol, and phosphate buffer were determined with the Cenco DuNouy Tensiometer. A lowered surface tension should lead

TABLE 9

COMPARISON OF PLATE COUNTS ON PURE CULTURE DILUTION WITH PHOSPHATE AND TRIS BUFFERS

	25 Plate Average Count	% Signif. Each Dil. Vs. Phos.-0	Signif. Change Vs. Phos.-0
Phosphate-0 Min.	88.0		
Phosphate-20 Min.	95.0	82	--
Phosphate-1 Hour	97.8	92	--
Phosphate-2 Hours	96.6	87	--
Phosphate-4 Hours	97.4	90	--
Phosphate-8 Hours	95.8	85	--
Phosphate-1 Day	58	99.5	-34%
Phosphate-2 Days	41	99.5	-53%
Tris-0 Minutes	93.2	80	--
Tris-20 Minutes	98.8	94	--
Tris-1 Hour	91.6	70	--
Tris-2 Hours	91.7	70	--
Tris-4 Hours	106.4	98	+25%
Tris-8 Hours	141.0	99.5	+60%
Tris-1 Day	1890	99.5	+2050%
Tris-2 Days	20,000	99.5	+21000%

TABLE 10

COMPARISON OF SIGNIFICANT CHANGES OF TRIS AT FOUR HOURS AND TRIS AT EIGHT HOURS WITH PHOSPHATE AT ZERO MINUTES

	Plate Counts Expressed As:				
Compound	Average	Median	Mode	Ave. Phos. & Tris	%Signif. Each Tris Exposure Vs. Phosphate
Phosphate	88.0	84.0	76.0	-	-
Tris 4-Hr.	106.4	103.0	109.1	97.2	98
Tris 8-Hr.	141.0	135.0	147.0	114.5	99.5
% Increase of Tris 4-Hr. Over Phosphate	25.5	22.6	43.4		
& Increase of Tris 8-Hr. Over Phosphate	60.0	61.0	93.4		
Ratio of % of Counts Above Ave. of Phos. & Tris 4-Hr.				37.5/58.3	
Ratio of % of Counts Above Ave. of Phos. & Tris 8-Hr.				28.8/67.1	

TABLE 11

COMPARISON OF PLATE COUNTS OF HOMOGENIZED MILK USING PHOSPHATE AND TRIS BUFFERS

Compound	Time of Exposure	Average of Ten Plates	
		First Sample	Second Sample
Phosphate	0	20	251
Phosphate	20	24	239
Tris	0	19	257
Tris	20	19	246

to a greater dispersion affect. The values recorded were: distilled water--67.4 dynes/cm.; phosphate buffer--65.2 dynes/cm.; t-butyl alcohol--63.4 dynes/cm.; and Tris--55.1 dynes/cm.

DISCUSSION

Although the counts increased in Tris after a day or two to such an extent, that growth and reproduction was obvious, this effect must be discounted when there has been only twenty minutes of contact between the organisms and Tris buffer. Twenty minutes is about the shortest generation time for any organism and can be attained only when the organism has been growing in optimal medium and is being transferred to another optimal medium. In fact, most organisms when isolated from an attenuating environment such as river water need at least an hour or two (lag phase) before the growth and reproduction phase begins. This means, therefore, that the increased counts after standing in Tris buffer for twenty minutes must be due to some other factor than cell division.

Increases may be due to dispersion action of the Tris. In other words, a bacterial clump would act as though it were only one organism when counted by plate count or multi-tube serial dilution methods, after being diluted in phosphate buffer while Tris and t-butyl alcohol might tend to break these clumps into individual cells with the resulting higher counts.

The higher counts using Tris and (t-butanol also) as compared to phosphate buffer could also be due to

the protective effect of the organic compounds. The protective effect is not a new idea. As early as the 1930's Huddleson and coworkers (1960) were using 0.05% tryptose-saline as a standard laboratory diluent because of the protective effect of the tryptose. They found that several proteinaceous substances gave a protective effect to Brucella organisms while phosphate buffer, saline and distilled water gave rise to lower counts.

Lipe and Mallmann (1960) used Dubos Medium (protein solution) as a diluent as well as a nutritive source.

The protective effect is further substantiated in the results of comparisons of pure culture and homogenized milk dilutions. Since there is not any significant difference regardless of which diluent is used in these two cases, the similarity in counts probably is due to the protective action of the protein compounds from the milk and the pure culture broth.

The second reason for similar counts regardless of diluent with milk and pure culture, is no doubt due to the fact that the organisms are in a fairly optimum growth environment and not subject to attenuation such as occurs with river organisms.

It must not be forgotten that Pacheco and Dias (1954) felt that phosphates liberate an autolytic enzyme which attack the coliforms. This is a plausible answer for any differences which may arise between counts after phosphate dilution and after organic buffer dilution, but it cannot be accepted as the only answer, if at all.

Any decreased counts (as compared to phosphate) such as t-butanol-20 minutes, hydroxymethyl-methyl-propanediol-20 minutes, and both nitro-propanediols at both times must be due to an inherent toxicity to coliforms in the particular compound.

Amino-methyl propanediol and dimethyl propanediol would be about as satisfactory as phosphate except perhaps in expense and time to prepare.

Thus it would seem that the beneficial effects of Tris are probably due to both dispersion and "protective colloid", while phosphate offers neither action and is possibly even slightly antagonistic to most bacteria.

SUMMARY

A study was made on the effect of various diluents on river coliform indices. Tris and structural analogues of it were compared to phosphate buffer. Tris, t-butyl alcohol, and hydroxymethyl-methyl propanediol gave "zero-time" counts which were higher than phosphate, but Tris was the only one in which counts stayed consistently high upon standing. Amino-methyl and dimethyl propanediols gave very similar counts to phosphate. The nitro-propanediols gave lower counts than phosphate buffer.

There appeared to be no difference among the diluents as compared to phosphate buffer when the organism source was homogenized milk or pure laboratory cultures.

The beneficial effects of Tris seem to be due to a combination of "protective colloid" and "dispersion effect". There is also a possibility that phosphate is slightly unfavorable toward coliforms, thus enhancing the value of Tris.

It is recommended that Tris replace Butterfield's Buffer as a diluent for quantitative bacteriological work.

CONCLUSIONS

1. Tris is superior to phosphate buffer for diluting coliforms, whether the dilution is made immediately or not.
2. Tris probably exerts both a protective action and a dispersion effect on attenuated coliforms.
3. t-butyl alcohol and 2-hydroxymethyl-2-methyl-1,3-propanediol are superior to phosphate buffer for diluent purposes only if the dilution is made and tubed immediately.
4. 2-amino-2-methyl-1,3-propanediol and 2,2-dimethyl-1,3-propanediol are very comparable to phosphate buffer as dilution waters.

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