A COMPARATIVE STUDY OF MERTHIOLATE, PHENOL, AND SEVERAL QUATERNARY AMMONIUM COMPOUNDS AS PRESERVATIVES FOR SERUM

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THESIS

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by

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INTRODUCTION

The preservation of serum in the liquid state is of paramount importance in the field of therapeutic medicine. Preservation in this form is simple, economical, and provides the phlebotomist with an instantly available form of the blood derivative. The use of serum and plasma played a significant part in the treatment of casualties in World War II and in civilian defense. Many attempts have been made to find a preservative which could be added to the serum in a concentration that would be bacteriostatic and yet remain non-toxic. The establishment of the quaternary ammonium compounds as excellent bactericides and bacteriostats, and the fact that they are relatively non-toxic in use-dilutions, stimulated the undertaking of this work. The two major difficulties encountered in preserving serum in the liquid state are: (a) rapidity of bacterial growth when contaminated, and (b) precipitation and loss of essential elements.

At present, there are four common methods for the preservation of serum: (a) simple desiccation; (b) storage in the liquid state with or without chemical preservatives; (c) vacuum desiccation from the frozen state; and (d) preservation in the frozen state. Each method has its own advantages and disadvantages.

REVIEW OF THE LITERATURE

Emerson (4), in 1923, introduced a method of preserving

plasma and serum by subjecting it to high pressures. A chamber was built to withstand considerable pressure. The material was introduced into the chamber and subjected to concussive impact by the action of a plunger. Since that time, it has become evident that microorganisms are capable of withstanding pressure far greater than is practicable to apply, and that under those conditions denaturation of the original serum proteins would occur.

Harper (13) offered a method of preserving serum by simple desiccation. The material is dried in a vacuum distilling apparatus at reduced pressure (about 15 mm mercury) between 40°-45°C. It was found that the serum remained stable in this form over long periods of time.

Strumia (27) described a method for preserving serum and plasma in the frozen state. The technique involved three essential features; (a) fairly rapid freezing, (b) maintenance at a temperature well below the melting point (-5° to -78°C), and rapid thawing in a water bath at 37°C and warming at room temperature.

Flosdorf and Mudd introduced the "Lyophile" (7) and "Cryochem" (8) processes for the preservation of biological materials (serum, plasma, etc.). The procedures are essentially the same with the exception of the method of elimination of water vapor. The material is first frozen and placed in a vacuum apparatus. In the Lyophile process the water vapor is condensed as ice in a container kept cold by

dry-ice. In place of dry-ice, the Cryochem equipment utilizes a regenerative chemical desiccant - Calcium sulfate.

Noble (19) used varying dilutions of glycerol, chinsol, phenol, chloroform, mercuric iodide, dibromin, ortho-chlorophenol, trikresol, and chloretone for preserving agglutinating serum. He showed that the sterility of the individual samples was retained over a period of four years at 6°-8°C.

Other chemical agents which have been used as preservatives for serum are quinsol, formalin, carvasept, eucupin, hexylresorcinol, and yatrene (an iodine derivative of pyridine). The organic mercury compounds are among the more common chemical agents now used to preserve sera. These will be discussed more thoroughly later. Gershenfeld (10) stated that "mercurials appear to be unsuited in the preservation of sera to be concentrated and refined. The composition and amount of the preservative are important. Liquid serum must not contain more than 0.5 percent of preservative, nor more than 20 percent total solids. Products intended for intravenous use must be clear, free from excessive coloration and viscosity."

Novak (20) made a comparative study of the sulfone derivatives (sodium sulfathiazole, sodium sulfapyridine, and sulfanilamide) for preserving plasma. He concluded that a final concentration of 0.2 percent sodium sulfathiazole solved the problem of gross bacterial contamination in plasma, and that actual sterilization of the material occured when there

was minimum contamination. He also demonstrated that it was desirable to add the sulfonamide compounds to plasma and maintain it at room temperature for several days previous to storage in the refrigerator. If the plasma was placed directly into the refrigerator, a longer period of time was required and sterilization occured only in the higher concentrations. This was, no doubt, due to the reduced activity of the sulfone derivatives at lower temperatures.

DESCRIPTION OF COMPOUNDS

A description of the compounds tested and a review of the literature regarding their use as applicable to this work is as follows:

Merthiolate

Merthiolate - sodium ethyl mercuri thiosalicylate - is produced by the Eli Lilly and Company, and is marked "Released for use as a biological preservative".

Jamieson and Powell (16) noted that merthiolate was free of precipitating properties when mixed in strong concentrations with protein solutions, and that under these conditions maintained its germicidal properties. They also noted that it had a low degree of toxicity and was harmless toward labile antigen fractions and antibodies. MacKay (17) showed that a 1-10,000 dilution of merthiolate in human serum prevented the growth of Staphylococcus aureus and Bacillus subtilis. Studies with the Agar-cup Plate Method by Rose and Miller (24) indicated that the effectiveness of merthiolate diminished as

the blood concentration increased. Smith, Czarnetzky, and Mudd (26) found that mercurial antiseptics exert their action almost wholly by virtue of their mercury content, and that the killing power per unit weight of mercury falls off rapidly with dilution when used in the presence of serum. Fildes (6) demonstrated that mercury combines with -SH groups in the cell, thus depriving the cell of R-SH groups which are essential for its metabolism. Hoogerheide (14) demonstrated that the critical bacteriostatic dilution of merthiolate against Staph. aureus was not changed in the presence of 10 percent serum.

Phenol

In testing merthiolate (1-10,000) and phenol (1-200), Rosenstein and Levin (25) noted that the failure of the preservative to destroy the organism tested could not be attributed to the lessening of the potency of the preservative due to storage in the presence of serum. It was also shown that the efficiency of phenol against staphylococci, B. pyocyaneous, and diphtheroids decrease during 10 month storage at 5°C, and that maximum effect of the preservative on the organism is usually reached in 48 hours. Falk and Aplington (5) showed that the effect of using phenol and merthiolate in mixtures is additive. Hoogerheide (14) noted that the critical bacteriostatic dilution of phenol against Staph. aureus was reduced from 1-450 to 1-280 in the presence of 10 percent serum.

Quaternary Ammonium Compounds

There is an extensive literature presenting evidence to show that the high molecular quaternary ammonium compounds (cationic) are effective antiseptics and disinfectants. An excellent review of the literature concerning their chemical structure, physical properties, and interactions between proteins is presented by Glassman (11). The author was not able to find any literature concerning their use as preservatives for serum. The specifications adopted by the Food and Drug Administration utilizing 10 percent normal horse serum as organic matter in testing disinfectants, have supplied much information regarding the effect of serum on the germicidal potency of quaternary ammonium compounds and the effect of the quaternary ammonium compounds on the serum.

Anson (1) showed that proteins may be denatured by surface active agents of either anionic or cationic type. He attributed the denaturation to the type of hydrophobic-hydrophilic structure. Putnam and Neurath (22) noted that precipitation of serum proteins appeared to be caused by electrostatic forces. The maximum concentration of the quaternary ammonium compound required for complete precipitation corresponded closely to the total acid binding capacity of the proteins.

Hotchkiss (15) demonstrated that surface active cations cause an irreversible damage to the cellular membrane of bacteria, which results in the release of soluble nitrogen and phosphorus compounds from the cell and the death of the cell.

BTC

BTC - a mixture of alkyl-dimethyl-benzyl-ammonium chlorides.

Using Salmonella typhosa, Harley (12) noted that alkyl-dimethyl-benzyl-ammonium chloride had its killing dilution reduced from 1-30,000 to 1-6,000 in the presence of 10 percent serum. Hoogerheide (14) demonstrated that the critical bacteriostatic dilution of this compound against Staph. aureus was reduced from 1-400,000 to 1-140,000 in the presence of 10 percent serum.

Tetrosan

Tetrosan - alkyl-dimethyl-3-4-dichlorobenzyl-ammonium chloride.

DuBois and Dibble (3) noted that the killing dilution of Tetrosan against Staph. aureus, when determined by the F.D.A. method was approximately 1-50,000.

Hyamine #2389

Hyamine #2389 is a derivative of Phemerol (p-tertiary-octyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl-ammonium chloride), having essentially the same bactericidal and bacteriostatic effect on microorganisms. No information was available on Hyamine #2389, however, there was on Phemerol. Mallmann (18) noted that the killing dilution of Phemerol against Sal. typhosa was reduced from 1-22,000 to 1-5,200 in the presence of 10 percent serum. Hoogerheide (14) noted that the critical bacteriostatic dilution of Phemerol against Staph. aureus was

reduced from 1-400,000 to 1-140,000 in the presence of 10 percent serum.

Cetyl-pyridinium chloride

For brevity, cetyl-pyridinium chloride will be referred to as CPCl for the remaining part of this work.

Quisno and Foter (23) showed that 10 percent serum reduced the germicidal activity of CPCl from 12 to 25 times when tested against gram negative organisms. Hoogerheide (14) noted that the critical bacteriostatic dilution of this compound when tested against Staph. aureus, was reduced form 1-1,000,000 to 1-420,000 in the presence of 10 percent serum. Pharmacological and toxicological studies carried out by Warren et el (28) on rabbits, revealed that the MLD of CPCl was 20 mgm per kgm of body weight, and the LD_{50} was 35 mgm per kgm of body weight.

Isothan Q-15

Isothan Q-15 - lauryl isoquinolinium bromide.

Information on this compound was obtained directly from the Onyx laboratories (21). The killing dilutions of Isothan Q-15 in the absence of serum were found to be 1-7,000 for Escherichia coli and 1-20,000 for Staph. aureus. Toxicological studies on guinea pigs showed that the MLD was 80 mgm per kgm of body weight, and the LD_{50} was 200 mgm per kgm of body weight.

EXPERIMENTAL AND DISCUSSION

Normal horse serum was obtained from the Michigan Department of Health, Lansing, Michigan. Three Lots* were required for this

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* Lot #652 - 2,800 ml
Lot #C-654 - 1,400 ml
Lot #A-644 - 1,400 ml

work. Prior to delivery the serum was passed through a bacteriological filter and collected in a sterile one gallon glass jug. The serum was then dispensed under aseptic conditions into sterile screw-cap test tubes (25 ml). These tubes were kept in the refrigerator (2°-10°C) until ready for use. Representative samples were added to Brewer-Modified Thioglycollate medium (2) to check for sterility. The tubes were incubated for 48 hours at 37°C. No contamination was found.

The organisms used in this work were Micrococcus pyogenes var. aureus #209 and Escherichia coli #9637. All cultures used in this study were 24 hour slant cultures which had been transferred at least three successive days on plain nutrient agar slants. The 24 hour slant cultures were washed with saline and diluted to compare with a set of standard nephelometer tubes. The desired number of organisms was obtained by diluting with sterile saline. A check on the final number of organisms was made by plating 1 ml portions of the dilution on four Tryptose Glucose Extract (T.G.E.) agar plates. An average count was obtained from the four plates.

An attempt was made, not to determine the exact amount of compound necessary to kill the organisms in the presence of serum, but to determine if the organisms were killed or inhibited by the preservatives at concentrations compatible with the serum and within the limits of safety used in biological products for regular distribution.

All dilutions of the compounds and serum are reported in terms of final concentration.

For convenience, stock solutions were prepared by diluting with sterile distilled water. These dilutions were kept in screw-cap test tubes at room temperature. The dilutions prepared were as follows: (a) Merthiolate 1-250, 1-500, and 1-1,250. A 1-10,000 dilution of merthiolate had a pH of 6.75. (b) Phenol 1-20 and 1-50. A 1-200 dilution of phenol had a pH of 6.8. (c) BTC 1-250, 1-500, and 1-1,250. A 1-10,000 dilution of BTC had a pH of 7.0. (d) Tetrosan 1-250, 1-500, and 1-1,250. A 1-10,000 dilution of tetrosan had a pH of 6.75. (e) Hyamine #2389 1-250, 1-500, and 1-1,250. A 1-10,000 dilution of Hyamine #2389 had a pH of 6.95. (f) CPCl 1-500 and 1-1,250. (g) Isothan Q-15 1-250, 1-500, and 1-1,250. A 1-10,000 dilution had a pH of 5.55.

The first phase of this study consisted of testing the compatibility of the various compounds with serum at different temperatures. Two sets of tubes containing 10 ml of serum served as controls. To sterile cotton plugged test tubes were added 9 ml of serum and 1 ml of the proper dilution of compound. Individual sets were incubated at refrigerator (2°-10°C), room (22°-24°C), and 37°C temperatures. The results are presented in Tables I and II.

From these data it is apparent that refrigerator temperatures are more compatible with the serum-compound mixtures than either room or 37°C temperatures. The concentration of the various compounds seemed to have little or no visible effect on the serum proteins at these temperatures, with the exception of the

TABLE I

The Precipitation of Normal Horse Serum at Various Temperatures.

Control Tubes	Temperature								
	2°-10°C			22°-24°C			37°C		
	Time in Days								
	1	5	15	1	5	15	1	5	15
#1	-	-	-	-	SP	P	SP	P	P
#2	-	-	-	-	SP	P	SP	P	P

(-) No visible precipitate
(SP) Slight precipitate
(P) Precipitate

TABLE II

Compatibility of Compounds with 90 Percent Normal Horse Serum at Various Temperatures.

Temperature	2°-10°C									22°-24°C									37°C								
Dilution	1-5,000			1-10,000			1-50,000			1-5,000			1-10,000			1-50,000			1-5,000			1-10,000			1-50,000		
Time in Days	1	5	15	1	5	15	1	5	15	1	5	15	1	5	15	1	5	15	1	5	15	1	5	15	1	5	15
Merthiolate	-	-	-	-	-	-	-	-	-	-	SP	P	-	SP	P	-	SP	P	SP	P	P	SP	P	P	SP	P	P
BTC	-	-	-	-	-	-	-	-	-	-	SP	P	-	SP	P	-	SP	P	SP	P	P	SP	P	P	SP	P	P
Tetrosan	-	-	-	-	-	-	-	-	-	-	SP	P	-	SP	P	-	SP	P	SP	P	P	SP	P	P	SP	P	P
Hyamine #2389	-	-	-	-	-	-	-	-	-	-	SP	P	-	SP	P	-	SP	P	SP	P	P	SP	P	P	SP	P	P
CPCl	ND			-	-	-	-	-	-	ND			-	SP	P	-	SP	P	ND			SP	P	P	SP	P	P
Isothan Q-15	-	-	-	-	-	-	-	-	-	SP	P	P	-	SP	P	-	SP	P	P	P	P	SP	P	P	SP	P	P
Dilution	1-200			1-500			1-1,000			1-200			1-500			1-1,000			1-200			1-500			1-1,000		
Phenol	-	-	-	-	-	-	-	-	-	-	SP	P	-	SP	P	-	SP	P	SP	P	P	SP	P	P	SP	P	P

(-) No visible precipitate
(SP) Slight precipitate
(P) Precipitate
(ND) Not determined

1-5,000 dilution of Isothan Q-15, which seemed to enhance precipitation.

A preliminary experiment was carried out to determine the effect of sodium thiosulfate on merthiolate at bacteriostatic dilutions. A medication tube containing 18 ml of serum, 1 ml of merthiolate (1-250), and 1 ml of diluted M. pyogenes var. aureus culture containing 58,000 organisms was mixed and allowed to stand for five minutes. Four 1 ml portions were pipetted into four test tubes containing 9 ml of F.D.A. broth and mixed. One ml was transferred to another tube containing 9 ml of F.D.A. broth and another 1 ml portion was transferred to a tube containing 4.5 ml of sodium thiosulfate (1-50) and 4.5 ml of F.D.A. broth. All tubes were incubated at 37°C for 48 hours. The results are presented in Table III.

The results of this preliminary test showed that sodium thiosulfate was capable of exerting a neutralizing effect on merthiolate at the bacteriostatic dilution (1-500,000). For the remainder of this work sodium thiosulfate was used to neutralize the effect of merthiolate in the second dilution tube.

The second phase of this work involved a method similar to that used by Smith, Czarnetzky, and Mudd (26) for determining the germicidal activity of mercurials in the presence of serum. Only those modifications were made which were necessary to meet the demands of this experiment. The purpose of this experiment was to determine the germicidal activity of the compounds in the presence of serum over a 90 minute period. In most cases

TABLE III

Neutralizing Effect of Sodium Thiosulfate on Merthiolate (1-500,000) against M. pyogenes var. aureus* in the Presence of 1 Percent Normal Horse Serum.

Tube Number	#1 Dilution	#2 Dilution	
	F.D.A. Broth	F.D.A. Broth	$Na_2S_2O_3$** / F.D.A. Broth
1	-	-	/
2	-	-	/
3	-	-	/
4	-	-	/

* Number of organisms used in test - 58,000
** 4.5 ml of sodium thiosulfate (1-50) was added to 4.5 ml of F.D.A. broth.
(-) No visible growth
(/) Visible growth

a final concentration of 90 percent normal horse serum was used. This facilitated the amount of preservative and diluted organisms to be added, and also provided a total volume from which representative samples could be taken without introducing a large error. Thus, to 18 ml of serum were added 1 ml of preservative and 1 ml of diluted culture. Owing to the limited solubility of phenol, it was necessary, in order to obtain a final volume of 20 ml, to use 17 ml of serum, 2 ml of phenol (1-20), and 1 ml of the diluted culture, resulting in a final 85 percent serum mixture and a 1-200 dilution of the compound. Individual medication tubes were prepared containing 18 ml of serum. To these were added respectively, 1 ml of merthiolate (1-250), 1 ml of BTC (1-250), 1 ml of Tetrosan (1-250), 1 ml of Hyamine #2389 (1-250), 1 ml of CPCl (1-500), and 1 ml of Isothan Q-15 (1-250). All tubes were placed in a water bath maintained at room temperature (22°-24°C) during the time of exposure. At the initial time, 1 ml of the diluted culture of M. pyogenes var. aureus was added to the medication tube and thoroughly mixed with the serum and preservative. At intervals of 1, 5, 10, 15, 30, 60, and 90 minutes, a 1 ml portion of the mixture was removed, pipetted into a sub-culture tube containing 9 ml of F.D.A. broth and mixed. One ml of this mixture was then transferred into another tube containing 9 ml of F.D.A. broth and mixed. From this second tube four Petri dishes received 1 ml portion each. Ten ml of melted T.G.E. agar (48°C) was poured into the plates, mixed thoroughly and allowed to solidify.

All plates were incubated at 37°C for 48 hours. Sodium thiosulfate was used in dilution tube #2 to neutralize the action of merthiolate. For the other compounds, the #2 dilution tubes contained only F.D.A. broth. The F.D.A. broth tubes were used to dilute the compounds beyond their bacteriostatic ranges. Thus the absence of bacterial colonies on the plates would indicate germicidal activity. Results of this experiment are presented in Tables IV - X.

The results of this experiment showed that the compounds were not able to kill the organisms in the presence of serum over a period of 90 minutes. Bacteriostasis was observed in many of the dilution tubes. Garrod (9) noted that the bacteriostatic concentration varied according to the number of organisms present. This explains the difference in observable growth obtained in the dilution tubes. The number of organisms used in the test was kept at a minimum to facilitate plate counts, saving both time and materials.

The third phase of this work was to determine if the germicidal activity of these compounds in the presence of serum could be detected on incubation of the mixtures at 37°C for periods of 3, 7, and 25 days against M. pyogenes var. aureus and E. coli.

Two sets of screw-cap test tubes were prepared containing serum, compound, and organisms. One set was inoculated with M. pyogenes var. aureus and the other with E. coli. A separate set was prepared containing only the serum and organisms; these served as controls. Duplicate sets of tubes were prepared contain-

TABLE IV

Effect of 1-5,000 Merthiolate on M. pyogenes var. aureus* in the Presence of 90 Percent Normal Horse Serum at 22°-24°C.

Time of Exposure in Minutes		1	5	10	15	30	60	90
Dilutions in F.D.A. Broth	#1 1-50,000	-	-	-	-	-	-	-
	#2 1-500,000**	≠	≠	≠	≠	≠	≠	≠
Average Count of Four T.G.E. Plates		25	31	23	25	22	23	25

* Number of organisms used in test - 132,000
** A 1-100 dilution of sodium thiosulfate in F.D.A. broth.
(-) No visible growth
(≠) Growth

TABLE V

Effect of 1-200 Phenol on M. pyogenes var aureus* in the Presence of 85 Percent Normal Horse Serum at 22°-24°C.

Time of Exposure in Minutes		1	5	10	15	30	60	90
Dilutions in F.D.A. Broth	#1 1-2,000	≠	≠	≠	≠	≠	≠	≠
	#2 1-20,000	≠	≠	≠	≠	≠	≠	≠
Average Count of Four T.G.E. Plates		20	18	23	22	20	21	19

* Number of organisms used in test - 44,000

TABLE VI

Effect of 1-5,000 BTC on M. pyogenes var. aureus* in the Presence of 90 Percent Normal Horse Serum at 22°-24°C.

Time of Exposure in Minutes		1	5	10	15	30	60	90
Dilutions in F.D.A. Broth	#1 1-50,000	-	-	-	-	-	-	-
	#2 1-500,000	-	-	-	-	-	-	-
Average Count of Four T.G.E. Plates		24	21	19	20	23	21	21

* Number of organisms used in test - 52,000

TABLE VII

Effect of 1-5,000 Tetrosan on M. pyogenes var. aureus* in the Presence of 90 Percent Normal Horse Serum at 22°-24°C.

Time of Exposure in Minutes		1	5	10	15	30	60	90
Dilutions in F.D.A. Broth	#1 1-50,000	-	-	-	-	-	-	-
	#2 1-500,000	-	-	-	-	/	/	/
Average Count of Four T.G.E. Plates		19	20	21	18	19	22	22

* Number of organisms used in test - 52,000

TABLE VIII

Effect of 1-5,000 Hyamine #2389 on M. pyogenes var. aureus* in the Presence of 90 Percent Normal Horse Serum at 22°-24°C.

Time of Exposure in Minutes		1	5	10	15	30	60	90
Dilutions in F.D.A. Broth	#1 1-50,000	-	-	-	-	-	-	-
	#2 1-500,000	-	-	-	-	-	-	-
Average Count of Four T.G.E. Plates		18	18	19	20	20	17	19

* Number of organisms used in test - 43,000

TABLE IX

Effect of 1-10,000 Cetyl-pyridinium chloride on M. pyogenes var. aureus* in the Presence of 90 Percent Normal Horse Serum at 22°-24°C.

Time of Exposure in Minutes		1	5	10	15	30	60	90
Dilutions in F.D.A. Broth	#1 1-100,000	+	+	+	+	+	+	+
	#2 1-1,000,000	+	+	+	+	+	+	+
Average Count of Four T.G.E. Plates		35	38	40	41	46	52	31

* Number of organisms used in test - 65,000

TABLE X

Effect of 1-5,000 Isothan Q-15 on M. pyogenes var aureus* in the Presence of 90 Percent Normal Horse Serum at 22°-24°C.

Time of Exposure in Minutes		1	5	10	15	30	60	90
Dilutions in F.D.A. Broth	#1 1-50,000	-	-	-	-	-	-	-
	#2 1-500,000	-	-	-	-	-	-	-
Average Count of Four T.G.E. Plates		26	18	19	16	17	18	18

* Number of organisms used in test - 43,000

ing respectively, 1 ml of merthiolate (1-250) and 18 ml of serum; 2 ml of phenol (1-20) and 17 ml of serum; 1 ml of BTC (1-250) and 18 ml of serum; 1 ml of Tetrosan (1-250) and 18 ml of serum; 1 ml of Hyamine #2389 (1-250) and 18 ml of serum; 1 ml of CPCl (1-500) and 18 ml of serum; 1 ml of Isothan Q-15 (1-250) and 18 ml of serum; and one set containing 19 ml of serum to serve as a control.

To one set was added 1 ml of a diluted culture of M. pyogenes var. aureus containing 90,000 organisms per ml. To the other set was added 1 ml of a diluted culture of E. coli containing 275,000 organisms per ml. All tubes were incubated at 37°C. At intervals of 3, 7, and 25 days, 1 ml was removed, transferred to 9 ml of F.D.A. broth and mixed. One ml was removed from this dilution, transferred to a second tube containing 9 ml of F.D.A. broth and mixed. Sodium thiosulfate was used to neutralize the action of merthiolate. Four ml from the second dilutions was removed, 1 ml portions transferred to Petri dishes, mixed with melted T.G.E. agar (48°C) and allowed to solidify. Plates were incubated at 37°C for 48 hours. Results are presented in Table XI.

It was noted from the results in Table XI, that all of the compounds exhibited germicidal activity against M. pyogenes var. aureus in the presence of serum after contact for three days at 37°C. E. coli proved to be resistant to the action of the compounds over the entire period of 25 days with the exception of merthiolate. Merthiolate was the only compound to show any germicidal activity against E. coli.

TABLE XI

Effect of Compounds on the Organisms* in the Presence of 90 Percent Normal Horse Serum** at 37°C over a Period of 25 Days.

Compounds	Time in Days	M. pyogenes var. aureus			E. coli		
		F.D.A. Broth #1	F.D.A Broth #2	T.G.E. Plates	F.D.A. Broth #1	F.D.A. Broth #2	T.G.E. Plates
*** Merthiolate 1-5,000	3	-	-	-	-	-	-
	7	-	-	-	-	-	-
	25	-	-	-	-	-	-
Phenol 1-200	3	-	-	-	≠	≠	TNTC
	7	-	-	-	≠	≠	TNTC
	25	-	-	-	≠	≠	TNTC
BTC 1-5,000	3	-	-	-	≠	≠	TNTC
	7	-	-	-	≠	≠	TNTC
	25	-	-	-	≠	≠	TNTC
Tetrosan 1-5,000	3	-	-	-	≠	≠	TNTC
	7	-	-	-	≠	≠	TNTC
	25	-	-	-	≠	≠	TNTC
Hyamine #2389 1-5,000	3	-	-	-	≠	≠	TNTC
	7	-	-	-	≠	≠	TNTC
	25	-	-	-	≠	≠	TNTC
CPCl 1-10,000	3	-	-	-	≠	≠	TNTC
	7	-	-	-	≠	≠	TNTC
	25	-	-	-	≠	≠	TNTC
Isothan Q-15 1-5,000	3	-	-	-	≠	≠	TNTC
	7	-	-	-	≠	≠	TNTC
	25	-	-	-	≠	≠	TNTC
Control	3	≠	≠	≠	≠	≠	TNTC
	7	≠	≠	≠	≠	≠	TNTC
	25	≠	≠	≠	≠	≠	TNTC

* Number of organisms used in test:
M. pyogenes var. aureus - 90,000
E. coli - 276,000

** 85% Normal horse serum was used for phenol.

*** Sodium thiosulfate used in dilution #2 for merthiolate.

(-) No visible growth (≠) Visible growth

TNTC - Too numerous to count.

The fourth phase of this work was to determine if the germicidal activity of these compounds against M. pyogenes var. aureus and E. coli in the presence of serum at refrigerator temperatures (2°-10°C) could be detected.

All tubes were prepared in the same manner. To duplicate sets of screw-cap test tubes were added serum, compound, and organisms.

Two controls were run on each organism. To 19 ml of serum was added 1 ml of culture. The M. pyogenes var. aureus culture contained 48,000 organisms per ml. The E. coli culture contained 143,000 organisms per ml.

Phenol dilutions were prepared by adding to duplicate tubes, containing 17 ml of serum, 2 ml respectively of 1-20 and 1-50 phenol. One set was inoculated with 1 ml of M. pyogenes var. aureus culture containing 48,000 organisms per ml; the other set was inoculated with 1 ml of E. coli culture containing 143,000 organisms per ml.

Three tubes each were set-up for most of the remaining compounds. To each set containing 18 ml of serum per tube were added respectively, 1 ml of merthiolate (1-250), (1-500), and (1-1,250); 1 ml of BTC (1-250), (1-500), and (1-1,250); 1 ml of Tetrosan (1-250), (1-500), and (1-1,250); 1 ml of Hyamine #2389 (1-250), (1-500), and (1-1,250); 1 ml of CPCl (1-500) and (1-1,250); and 1 ml of Isothan Q-15 (1-250), (1-500), and (1-1,250).

To one set was added 1 ml of M. pyogenes var. aureus culture containing 29,000 organisms per ml; to the other set, 1 ml of E. coli culture containing 223,000 organisms per ml. Tubes were placed in the refrigerator and at intervals of 3,

5, 12, 25, and 50 days poured plates were made to determine bacterial survival.

One ml samples were removed from the tubes, transferred to 9 ml of F.D.A. broth and mixed. From these tubes 1 ml was transferred to a second tube containing 9 ml of F.D.A. broth and mixed. Sodium thiosulfate was added to the merthiolate tube. From the tubes containing M. pyogenes var. aureus 1 ml portions were pipetted from the second dilution tube into each of four Petri dishes. From the tubes containing E. coli one-half ml portions were pipetted from the second dilution tube into each of four Petri dishes. Ten ml of melted T.G.E. agar (48°C) was poured into the Petri dishes, mixed and allowed to solidify. All plates were incubated at 37°C for 48 hours.

Results of the control plates are presented in Table XII; those for phenol in Table XIII; and the results for the remaining compounds in Table XIV.

The results from this experiment indicated that the germicidal activity of all of the compounds, as compared to germicidal activity at 37°C, is greatly reduced at refrigerator temperatures. The control plates showed that M. pyogenes var. aureus was gradually being destroyed at this temperature. E. coli proved to be more resistant at this temperature, however, on the fiftieth day some reduction in number was noted. Merthiolate (1-5,000) proved to be the most active compound of the group, preventing growth on the M. pyogenes var. aureus plates on the twelfth day, and preventing growth on the E. coli

TABLE XII

Effect of Temperature (2°-10°C) on the Viability of Organisms* Grown in Normal Horse Serum.

Time in Days	*M. pyogenes* var. *aureus*		*E. coli*	
	Control Tube		Control Tube	
	#1	#2	#1	#2
	Average Count of Four T.G.E. Plates		Average Count of Four T.G.E. Plates	
3	29	23	71	72
5	19	21	71	81
12	8	16	65	73
25	7	9	67	70
50	3	5	50	50

* Number of organisms used in test:
M. pyogenes var. *aureus* - 48,000
E. coli - 143,000

TABLE XIII

Effect of Phenol (1-200) and (1-500) on the Viability of Organisms* in the Presence of 85 Percent Normal Horse Serum at Temperatures of (2°-10°C).

Time in Days	M. pyogenes var. aureus		E. coli	
	Average Count of Four T.G.E. Plates		Average Count of Four T.G.E. Plates	
	1-200	1-500	1-200	1-500
3	21	27	69	68
5	19	32	78	66
12	9	11	71	73
25	1	5	54	61
50	0	0	44	56

* Number of organisms used in test:
M. pyogenes var. aureus - 48,000
E. coli - 143,000

TABLE XIV

Effect of Various Dilutions of Compounds on Organisms* in the Presence of 90 Percent Normal Horse Serum at Temperatures of (2°-10°C).

	Time in Days	Merthiolate			BTC			Tetrosan			Hyamine #2389			CPCl			Isothan Q-15		
		1-5T	1-10T	1-50T	1-5T	1-10T	1-50T	1-5T	1-10T	1-50T	1-5T	1-10T	1-50T	1-5T	1-10T	1-50T	1-5T	1-10T	1-50T
M. pyogenes var. aureus	3	3	4	9	10	7	10	9	10	10	10	9	7	ND	6	9	7	12	14
	5	1	3	5	8	7	3	5	8	9	7	9	8	ND	7	10	5	7	12
	12	0	0	0	4	5	5	1	11	6	7	9	5	ND	5	10	2	7	9
	25	0	0	0	1	1	2	1	4	1	3	4	2	ND	2	5	0	3	8
	50	0	0	0	0	0	0	0	0	0	0	2	0	ND	0	2	0	0	0
E. coli	3	39	37	40	39	45	48	39	50	49	38	51	52	ND	50	39	46	43	53
	5	16	42	56	49	49	51	49	49	49	52	53	53	ND	55	51	35	41	58
	12	3	19	61	37	52	42	50	45	46	44	75	49	ND	60	48	37	38	47
	25	4	14	52	34	37	41	34	48	44	49	50	55	ND	56	55	32	38	44
	50	0	7	29	12	24	15	16	24	24	26	30	21	ND	35	19	30	31	33

* Number of organisms used in test:
M. pyogenes var. aureus - 29,000
E. coli - 223,000

(ND) Not determined

Note: Numbers represent the average count of four T-G.E. plates.

plates on the fiftieth day. A marked reduction in the number of E. coli was noted on the twelfth day in the 1-5,000 dilution of merthiolate. A similar reduction of E. coli was noted on the fiftieth day in the 1-10,000 dilution of merthiolate. The remaining compounds had little or no germicidal effect on either of the organisms at this temperature.

SUMMARY

A comparative study of merthiolate, phenol, and five quaternary ammonium compounds as preservatives for serum was made.

Dilutions of the compounds studied had little or no visible effect on the serum proteins. Temperature, in this case, appeared to be the influencing factor in the precipitation of the serum proteins.

Germicidal activity of the compounds studied could not be demonstrated when left for a contact period of ninety minutes with the test organism in the presence of serum.

On incubation of the serum, compound, and organism mixture at 37°C for three days, the germicidal activity of the compounds could be demonstrated.

At refrigerator temperatures (2°-10°C) the germicidal activity of the compounds is greatly reduced. Merthiolate was the only compound to exhibit germicidal action against both of the organisms tested. Merthiolate in concentrations of 1-5,000, 1-10,000, and 1-50,000 exhibited germicidal action against M. pyogenes var. aureus on the twelfth day of incubation

at refrigerator temperature. Merthiolate 1-5,000 exhibited germicidal activity against E. coli on the twelfth day, and in a concentration of 1-10,000 on the fiftieth day.

Merthiolate, as compared to phenol and the quaternary ammonium compounds tested, appeared to be the most satisfactory compound to use as a preservative for serum.

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