

THE PRESERVATION OF POLIOMYELITIS VACCINE WITH STABILIZED MERTHIOLATE

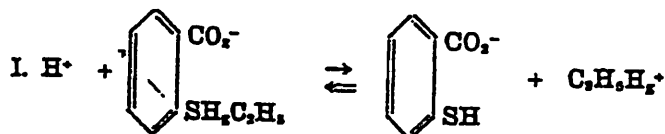
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INTRODUCTION

MERTHIOLATE* was first used as a biologic preservative in 1931.¹ Since that time, this antiseptic has been used for this purpose in a wide range of products including vaccines, toxoids, antigens of one kind or another, plasma, antisera, antitoxins, and others. Some of the properties of Merthiolate which contributed to its acceptance as a preservative in parenteral preparations are (1) water solubility, (2) compatibility with biologic materials, (3) relatively low toxicity, and (4) effectiveness as a growth inhibitor of bacteria and fungi.

Our present interest in re-examining the use of Merthiolate as a biologic preservative was stimulated by information from Dr. Jonas Salk² concerning the deterioration in antigenicity of some poliomyelitis virus vaccines which contained Merthiolate. When these preserved vaccines were subjected to increased temperatures in the incubator or to shipping conditions during the summer, the protective value of the vaccine was severely reduced in many instances. Although there was a significant variation of the effect of Merthiolate in the various vaccines, this preservative appeared to be deleterious to the vaccine. Either the Merthiolate had an unexpected direct action on poliomyelitis virus vaccine, or an unexpected set of conditions were being encountered in which Merthiolate was being degraded and was being rendered destructive to antigenicity.³

The antiseptic activity of Merthiolate (ethylmercurithiosalicylic acid, sodium salt) can be attributed to a partial ionization of the compound to give a low but effective level of ethyl mercuri ion ($C_2H_5Hg^+$), which blocks enzymatic processes by combining with sulfhydryl groups on the enzymes. This ionization is represented by the following equilibrium reaction:



The value of Merthiolate as a preservative over C_2H_5HgOH depends upon the fact that it provides a reservoir of $C_2H_5Hg^+$ at low concentration. The con-

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*Thimerosal, Eli Lilly Company.



centration of thiosalicylate ion obviously controls the level of free $C_2H_3O_2^-$ in the solution. A study of some of the factors which affect the stability of thiosalicylate and Merthiolate in solution has made it possible to solve the problem of the apparent incompatibility of Merthiolate and poliomyelitis vaccine.

MATERIALS AND METHODS

Merthiolate.—Merthiolate, 10 per cent aqueous solution, Mfg. Lot 606083, was kept in an amber-glass bottle under refrigeration as a stock solution.

Water.—Glass redistilled water was used for the physical and chemical studies in order to minimize the effects of trace metal ions.

Chemicals.—Analytical reagent grade chemicals were used for buffer salts and for reagents throughout, except as otherwise indicated. Triglycine and O,O' dicarboxydiphenyl disulfide were prepared by the Lilly Laboratories.

Various Monovalent and Trivalent Poliomyelitis Virus Vaccines.—These were supplied by the Biological Production Division of this company. The vaccines were prepared from the Mahoney, MEF1, and Saukett strains of virus propagated on monkey kidney tissue culture, with subsequent inactivation of the virus by Formalin. The residual formaldehyde in some vaccines was neutralized according to the methods used by Salk¹; such vaccines were referred to as "neutralized." The neutralization procedure was omitted in the preparation of the vaccines referred to as non-neutralized. Some vaccines, neutralized and non-neutralized, had Merthiolate added in various concentrations. Various vaccines containing Merthiolate included additives such as gelatin and glycine, whereas others had the trisodium salt of ethylene diamine tetra-acetic acid (EDTA). The vaccines were tested before and after heating at 37° C. for various periods, generally up to 14 or 15 days.

Assay of Merthiolate in Solution.—Relative concentrations of Merthiolate were determined by comparing the ultraviolet absorbance of the solution at 250 and 280 mμ with the spectrum of a Merthiolate solution of known concentration. This control sample was used in each series of measurements. The ultraviolet spectra were determined with a recording spectrophotometer.*

Effects of Trace Metal Ions on Merthiolate.—An accelerated test to study the effects of different compounds on Merthiolate simply required holding the sample at elevated temperatures. The buffered solutions (pH 7.0 potassium phosphate or pH 8.7 sodium bicarbonate) containing 0.01 per cent Merthiolate and the compound expected to influence its stability were sealed in glass ampules. The ampules were heated in a boiling water bath for specified times. After chilling, the samples were diluted and subjected to ultraviolet analysis. Appropriate control samples were used simultaneously.

Assay of Merthiolate in Polio Vaccine.—Detection of subtle changes in the Merthiolate in vaccine required its extraction from the vaccine. Five milliliters of vaccine was acidified with 0.2 ml. of 12 N HCl and extracted with 5 ml. of freshly distilled ether. After removal of the ether layer, the aqueous layer was again extracted with 2 ml. of ether. The combined ether solutions were shaken with 5 ml. of 1 per cent NaHCO₃. This bicarbonate solution was used for ultraviolet analysis.

Assays of Antigenicity.—In order to expedite immunologic tests of antigenicity of the various vaccines, considerable early use was made of the type 2 component and a one-dose mouse test. Information concerning the utility of this test was forwarded to us by Drs. Jonas Salk and Ulrich Krech,² prior to its formal publication by Krech.³ A similar test for types 1 and 3 was developed by Powell and Culbertson.⁴ Serum-virus neutralization tests against all three types of virus also were made in mice.⁵ These sera were obtained from mice 1 week after 3 weekly doses of vaccine.

The challenge viruses which we have used in the one-dose mouse immunity tests of different poliomyelitis vaccine preparations (for example, special Mahoney, MEF1, and Leon strains) have already been described.⁷ Mahoney, Lansing, and Leon strains were used in serum-virus neutralization tests conducted in mice.⁸

*Model 11 Cary.

Comparative Bacteriostatic and Fungistatic Action.—Some of the above lots of poliomyelitis vaccine preserved with Merthiolate in the presence of EDTA and glycine were utilized in comparative bacteriostatic and fungistatic tests. Test organisms included a laboratory strain of *Staphylococcus aureus* and four strains of fungi kept in these laboratories for use in chemotherapy tests. Such bacteriostatic and fungistatic tests were deemed necessary to prove the efficacy of Merthiolate as a preservative in the vaccine in the presence of these additives.

Effects of Thiomalicylate Ion on Fungicidal Activity of Merthiolate.—One hundred-ten-milliliter culture tubes containing sterile Sabouraud media were treated with 1 ml. of the test solutions before inoculation with a mixture of 13 yeasts. The tubes were left at room temperature and observed at intervals for signs of growth.

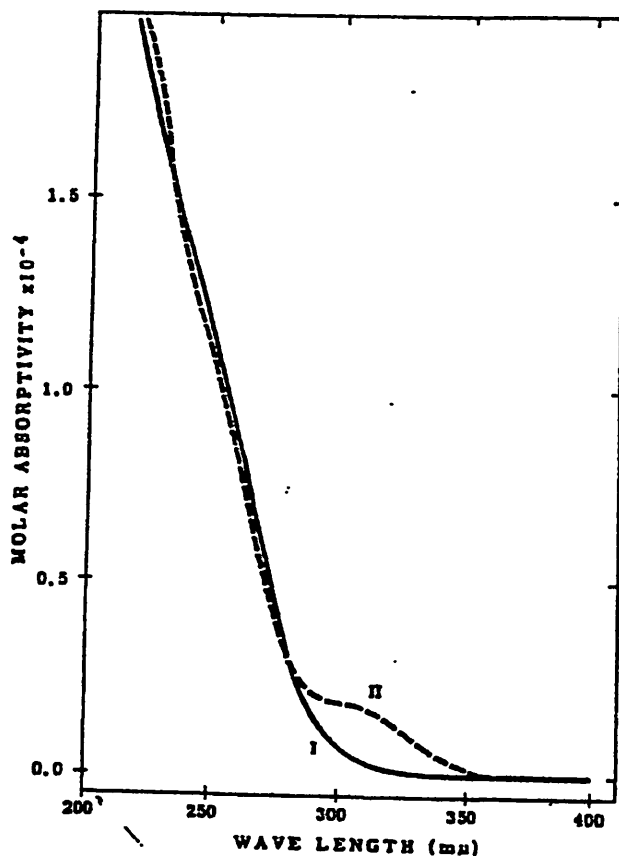


Fig. 1.—Ultraviolet spectra of 1.23×10^{-4} M aqueous Merthiolate solutions determined at pH 7 and 10 (Curve I) and at pH 2.0 (Curve II).

EXPERIMENTAL RESULTS

The Ultraviolet Absorption Curves of Merthiolate.—These are shown in alkaline, neutral, and acid solution in Fig. 1. Although the spectrum at neutral

pH does not have characteristic maxima and minima, the absorption is sufficiently intense to make ultraviolet absorption a feasible method of detecting Merthiolate in a solution if control solutions of known composition are used as standards.

The changes in the ultraviolet spectrum of Merthiolate resulting from heating the solutions in the presence of 1.0 p.p.m. Cu^{++} for various periods are illustrated in Fig. 2. This degradation of Merthiolate, as indicated by the reduced absorption near 250 $\text{m}\mu$, was also proportional to the amount of Cu^{++} present in the solution. Sufficient amounts of metal ions were present in glass

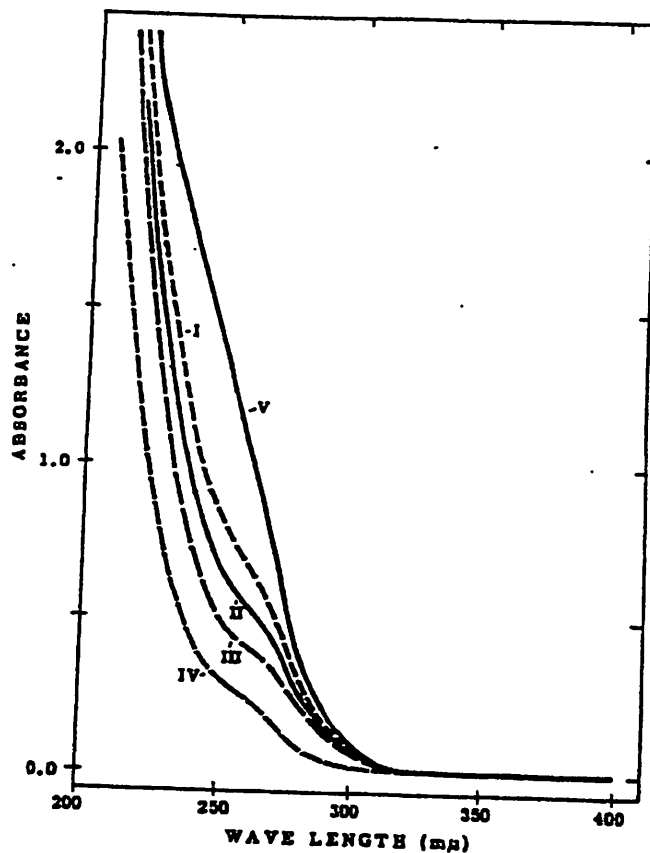


Fig. 2.—Effect of Cu^{++} on Merthiolate solutions. Changes in the ultraviolet spectrum of Merthiolate which demonstrate the chemical degradation of the compound. Merthiolate, $1.23 \times 10^{-3}\text{M}$, in 0.04M phosphate buffer, pH 7.0, and with 1 p.p.m. Cu^{++} was heated at 100°C . in sealed ampules for 5, 15, and 30 minutes (Curves I, II, and III, respectively). Curve V shows the spectrum before and after 30 minutes heating when 0.01 per cent EDTA was added to chelate the added Cu^{++} . Curve IV resulted from heating $1.23 \times 10^{-3}\text{M}$ $\text{C}_6\text{H}_5\text{H}_2\text{Cl}$ and $0.62 \times 10^{-3}\text{M}$ O,O' dicarboxyphenyl disulfide for 30 minutes.

redistilled water and analytical reagent grade chemicals to catalyze this breakdown at 100°C . The ferric ion had less than 1 per cent of the catalytic effect of the cupric ion.

The ultraviolet absorption curve of the degraded Merthiolate was essentially reproduced by warming a solution of ethylmercuric chloride and O,O'-dicarboxydiphenyl disulfide at pH 7.0. (Curve IV of Fig. 2.) This indicates that Cu^{++} catalyzes the oxidation of thiosalicylate to the disulfide under these conditions.

After the addition of stoichiometric quantities of EDTA to chelate the cupric ion, the Merthiolate remained stable in boiling water for prolonged periods. The ultraviolet spectra before and after 30 minutes of heating are reproduced as Curve V in Fig. 2. Although only one curve is shown, the spectrum of the heated sample coincided exactly with the unheated Merthiolate. Glycine afforded some protection against the effects of Cu^{++} but less than 5 per cent as effective as EDTA. Triglycine and gelatin were nearly inactive as stabilizing agents.

In order to determine the unchanged Merthiolate remaining in vaccines, the preservative was extracted from the acidified vaccine with freshly distilled ether. After shaking the ether solution with a bicarbonate solution, the aqueous phase was subjected to ultraviolet analysis. Typical ultraviolet spectra of samples extracted from vaccines subjected to different treatments are shown in Fig. 3. Freshly added Merthiolate was readily extracted from the vaccine (Curve I). When the preserved vaccine had been incubated for two weeks at 37°C ., very little Merthiolate remained in an ether extractable form (Curve II). In fact, the ether soluble material was very nearly the same as that from the fresh vaccine without preservative (Curve III), indicating that the thiosalicylic acid, the ultraviolet absorbing structure of Merthiolate, had been changed to an ether insoluble compound. The addition of the EDTA at the same time as the Merthiolate, however, prevented the accelerated breakdown of the preservative. The ultraviolet absorbing material of this sample (Curve IV) after three months at 37°C . was quite similar to that of freshly added Merthiolate (Curve I), but at a reduced concentration. Curve V was from a sample of the same vaccine as used for Curve IV, but this vaccine was stored for three months at 5°C .

Stability of Type 2 Vaccine Component in One-Dose Mouse Tests.—Information gained from numerous exploratory tests led to the establishment of comparative immunity and stability tests of the type 2 component in trivalent poliomyelitis vaccine treated under controlled conditions as follows: vaccine produced, inactivated, and neutralized in the regular wave was divided into three samples. The first sample was used as the control without further treatment. Merthiolate was added to the second sample at a concentration of 1:10,000 (0.000247 molar). To the third portion were added Merthiolate at 1:10,000 and EDTA, trisodium salt, at a concentration of 0.00247 molar. After various periods of heating at 37°C . to accelerate deterioration, single 0.5 ml. doses of the various vaccines in fivefold dilutions were injected intraperitoneally into different groups of 10 mice. Three days later, these mice were given a challenge dose of 0.1 ml. of special MEF1 poliomyelitis virus intravenously. This dose, established by previous titrations, was intended to be about 10 PD_{50} , and was sufficient virus to kill or paralyze from 7 to 10 of a group of 10 normal

mice, included in every test. Furthermore, one-tenth of this challenge dose was sufficient usually to kill from 2 to 6 of another group of 10 normal mice. Precise determination of exact number of PD₅₀ of virus used in each test is not necessary if the challenge dose is maintained in these limits. Seven days after the injection of the challenge virus, final readings were made and recorded in terms of mice appearing normal.

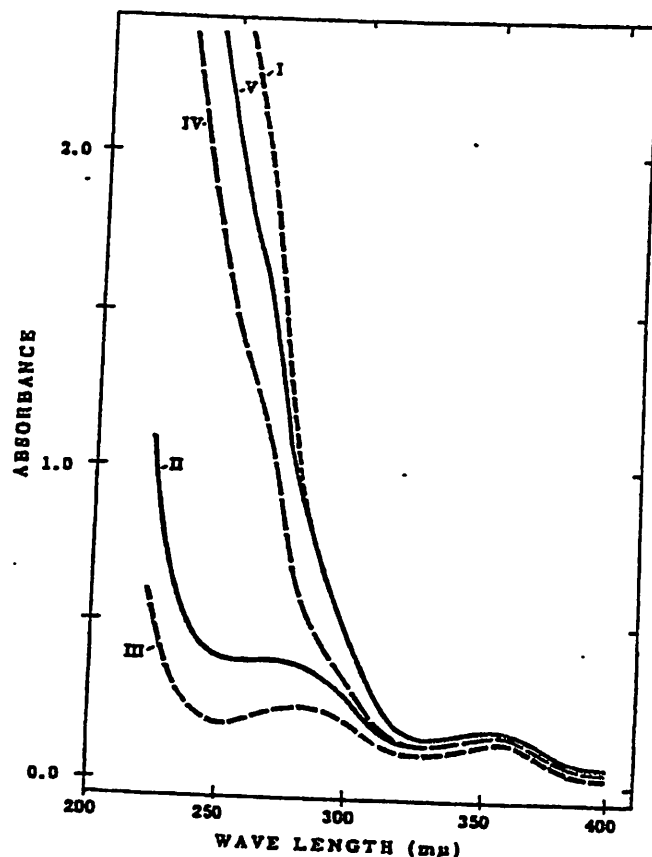


Fig. 1.—Stabilization of Merthiolate in vaccine. Ultraviolet spectra of bicarbonate solutions containing the ether-extractable material from acidified vaccines. When Merthiolate was used, the initial concentration was 0.01 per cent. Curve I. Freshly added Merthiolate. Curve II. Vaccine incubated 2 weeks at 37° C. with Merthiolate. Curve III. Unpreserved vaccine. Curve IV. Vaccine with Merthiolate and EDTA after 3 months at 37° C. Curve V. Vaccine with Merthiolate and EDTA after 3 months at 5° C.

Results of these first tests are shown in Table I. The unheated and undiluted vaccine protected 10 of 10 mice, and vaccine diluted 1:5 protected 5 of 10 mice against the challenge dose of virus which killed (or paralyzed) 9 of 10 controls. Samples of all three vaccines heated for 5 or 10 days, respectively, at 37° C. were included in this same test. It is noted from these results

that the vaccine sample preserved with Merthiolate 1:10,000 (vaccine 2684-B), has lost all of its type 2 potency after 10 days' heating. Vaccine without preservative (2684-A) and vaccine with Merthiolate and EDTA (2685-B) appear practically unaffected by heating. Subsequent tests of vaccine heated at 37° C. for eleven and fifteen days and certain repeat tests of vaccine heated fifteen days as indicated in Table I, gave the same general results; in other words, by using the type 2 component of trivalent vaccine as an index of stability, Merthiolate causes deterioration in potency, but the vaccine containing EDTA in addition to the Merthiolate immunizes as well as the unpreserved vaccine. Thus it became apparent in the early stages of this work that degradation products of Merthiolate, and not the Merthiolate molecule, were responsible for the loss of potency of the poliomyelitis vaccine.

TABLE I. STABILITY OF TYPE 2 COMPONENT OF TRIVALENT POLIOMYELITIS VACCINE ASSAYED BY ONE-DOSE MOUSE TEST

VACCINE	DILUTION	NORMAL MICE/MICE USED, SEVEN DAYS AFTER CHALLENGE						
		0 DAYS	5 DAYS	10 DAYS	11 DAYS	15 DAYS	15 DAYS (REPEAT)	15 DAYS (REPEAT)
2684-A	Str.	10/10	—	7/10	—	9/10	—	—
No Merthiolate	1:5	5/10	—	5/10	—	7/10	—	—
	1:25	—	—	—	—	—	4/10	6/9
	1:125	—	—	—	—	—	1/10	5/10
2684-B	Str.	—	—	1/10	—	1/10	—	—
Merthiolate 1:10,000	1:5	—	—	1/10	—	1/10	—	—
	1:25	—	—	—	—	—	—	—
	1:125	—	—	—	—	—	—	—
2685-B	Str.	—	7/10	—	8/10	—	5/9	—
Merthiolate 1:10,000	1:5	—	8/10	—	8/10	—	7/10	—
	1:25	—	—	—	—	—	—	6/10
	1:125	—	—	—	—	—	6/10	7/10
Virus virulence controls	1 challenge dose	1/10	1/10	1/10	0/10	0/10	2/10	3/10
	0.1 challenge dose	7/10	7/10	7/10	2/10	2/10	8/10	7/10

Different groups of 10 mice each received a single intraperitoneal dose of 0.5 ml. of the indicated vaccine dilutions and 3 days later were challenged intravenously with 0.1 ml. of 10⁻⁴ virus. The vertical columns indicate results with vaccines heated at 37° C. for various numbers of days.

Many different production lots of vaccine were tested for comparative type 2 response in mice using the same triad of samples (vaccine alone, vaccine with Merthiolate, and vaccine with Merthiolate and EDTA) and different periods of heating. These tests proved to be feasible and rapid in assessing various production methods in large scale preparation of vaccine.

Stability Tests of All 3 Components of Neutralized Trivalent Vaccine in N.I.H. Three-Dose Mouse Tests.—The results just described from the one-dose mouse tests with the type 2 component in trivalent poliomyelitis vaccine have been duplicated in the N.I.H. three-dose vaccine test in mice in which immunity against all 3 types of virus is determined. In these tests, groups of 20 mice are injected with 3 weekly intraperitoneal doses of 0.5 ml. of vaccine in fivefold dilutions, and one week after the third dose the mice are bled and the pooled

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sera from the groups are subjected to serum-virus neutralization tests in additional mice. Serum-virus dilutions involving types 1 and 3 are injected intraspinally into groups of 10 mice for each serum dilution; but for type-2 virus, the serum-virus dilutions are injected intracerebrally into similar groups of mice. Test doses of virus per mouse comprise about 10 PD₅₀ for types 1 and 3 and about 40 PD₅₀ for type 2. After final reading of the tests, the serum dilution saving 50 per cent of the mice is computed. Table II gives the results of this test applied to the same three heated vaccines tested previously by the one-dose test. In Table II it is noted that vaccine 2684-A after 15 days at 37° C. and without preservative immunizes mice sufficiently to show definite serum antibody titers to all three types of virus. Contrastingly, vaccine 2684-B heated with Merthiolate 1:10,000 does not immunize appreciably. However, when 0.00247 molar EDTA is added to Merthiolate 1:10,000 vaccine 2685-B, normal immunization of the mice against all three types of virus is attained with the heated vaccine. Thus, it has again been demonstrated that the addition of 10 moles of EDTA per mole of Merthiolate prevents the decrease in protective value of the poliomyelitis vaccine resulting from the addition of Merthiolate alone.

TABLE II. STABILITY OF TRIVALENT POLIOMYELITIS VACCINE ASSAYED BY MOUSE IMMUNIZATION AND SERUM NEUTRALIZATION TESTS*

VACCINE (HEATED 15 DAYS AT 37° C.)	THREE WEEKLY DOSES OF 0.5 ML. VACCINE DILUTED	SERUM-VIRUS NEUTRALIZATION TEST OF SERUM FROM MICE IMMUNIZED AS SHOWN IN COLUMN 2		
		(COMPUTED SERUM DILUTION PROTECTING 50% OF MICE)		
		TYPE 1	TYPE 2	TYPE 3
2684-A	Undiluted	18.8	6.3	8.6
No Merthiolate	1:5	7.5	5.6	5.7
2684-B	Undiluted	<4	<4	<4
Merthiolate 1:10,000	1:5	<4	<4	<4
2685-B	Undiluted	19.4	7.3	8.5
Merthiolate 1:10,000 + EDTA 0.00247 M	1:5	4.5	4.3	6.0

*Tests made as described by N. I. H. Minimum Requirements.

Stability of Trivalent Vaccine Including Different Concentrations of Merthiolate Without EDTA.—In similar experiments, it was observed that the type 1 component in neutralized trivalent poliomyelitis vaccine falls in potency somewhat faster in heat stability tests of vaccine preserved with Merthiolate alone than the types 2 and 3 components. At a concentration of 1:10,000, Merthiolate causes severe damage to the type 1 component and partial damage to the type 2 component after 5 days of heating at 37° C. Only slight loss in antigenicity of type 1 occurs during the same period if 1:20,000 Merthiolate is used, whereas 1:40,000, 1:50,000, and 1:100,000 are nearly without effect. However, if the period of heating is increased to 15 days, 1:100,000 Merthiolate causes appreciable loss of potency of the type 1 component.

Effects of Different Concentrations of EDTA.—In earlier experiments, 10 moles of EDTA were used for each mole of Merthiolate. Since the optimum

concentration of the stabilizing agent may be related to the amount of heavy metal ions in the solution and not to the amount or concentration of Merthiolate, experiments were conducted to determine the margin of safety which a concentration of EDTA of 0.00247 M would have in stability tests of 2 weeks at 37° C. under the conditions surrounding the preparation of vaccine in these laboratories. For this purpose, three fivefold decreasing concentrations of EDTA (0.00049 M, 0.000098 M, and 0.000019 M, respectively) were used in three lots of vaccine which were heated for 7 and 14 days at 37° C. Potency of the type 2 component in these three vaccines was determined by the one-dose vaccine test in mice with direct challenge 3 days later. The results are shown in Table III. If 5 to 10 mice of groups of 10 live, the vaccine is considered reasonably immunogenic. It appears, especially from the results in the 14 day column, that EDTA 0.000019 M, or 0.08 mole per 1 mole Merthiolate, is not sufficient, whereas 0.000098 M, or 0.4 mole EDTA per 1 mole Merthiolate is the minimal concentration of stabilizing agent necessary. This amount is one-twenty-fifth the amount used in the test described above; hence, there is a twenty-fivefold safety factor in terms of stability of the type 2 component in the vaccine. Since the type 1 component is somewhat more labile than the type 2, this safety factor would be less, but still adequate, for type 1. EDTA, 0.00247 M, alone added to neutralized vaccine has not been found to affect the potency of the vaccine when heated at 37° C. for 15 days.

TABLE III. EFFECT OF CONCENTRATION OF EDTA ON HEAT STABILITY OF TYPE 2 COMPONENT OF POLIOMYELITIS VACCINE PRESERVED WITH MERTHIOLATE 1:10,000

VACCINE	EDTA	MOLES EDTA PER MOLE MERTHI- OLATE	DIL. OF VACCINE	NORMAL MICE/MICE USED 7 DAYS AFTER CHALLENGE		
				0 DAYS	7 DAYS	14 DAYS
2751 (Parent lot)*	None (also no Merthiolate)	—	Str. 1:5 1:25	10/10 5/10 —	— — —	— — —
2759-A Merthiolate 1:10,000	0.00049 M	2	Str. 1:5 1:25	— — —	10/10 8/9 9/9	— 5/10 4/10
2759-B Merthiolate 1:10,000	0.000098 M	0.4	Str. 1:5 1:25	— — —	10/10 10/10 10/10	— 7/10 3/10
2759-C Merthiolate 1:10,000	0.000019 M	0.08	Str. 1:5 1:25	— — —	9/10 9/10 7/10	— 2/10 2/10
Virus virulence controls	1 challenge dose 0.1 challenge dose			1/10 7/10	2/10 7/10	1/10 5/10

One-dose mouse test used for assay. Columns at right show days of heating of vaccine at 37° C. prior to immunization.

*Same vaccine as 2584-A in Table I where heat stability is shown.

Gelatin at 1:1,000 and 0.3 M glycine were also tried as a protective agent for the vaccine. Although some stability was afforded by these compounds, 14 days of incubation at 37° C. caused an appreciable loss of activity in these vaccines.

Lack of Stability of Nonneutralized Vaccines Containing Merthiolate and EDTA. ~~The excess formaldehyde in all the vaccines referred to above had~~ been neutralized with sodium bisulfite. Vaccine preparations similar to those referred to above, but comprising "nonneutralized" samples, have been used in parallel stability tests similar to the heating experiment indicated with the neutralized vaccines. In contrast to the neutralized vaccine, the nonneutralized vaccines with the Merthiolate preservative and EDTA suffer a loss in potency in heat stability tests at 37° C. It is believed these results are so consistently negative, either at 7 or 14 days' heating, that detailed results need not be tabulated here. It is impractical to use nonneutralized nonpreserved poliomyelitis vaccine since the residual formaldehyde per se causes loss of antigenicity (first of the type 1 component, then of the other types) and this is readily demonstrable in stability tests conducted at 37° C. Furthermore, such vaccine does not show inhibitory action against mold growth.

On the Lack of Interference of EDTA on Antiseptic Action of Merthiolate.—Tests were conducted to determine if addition of EDTA along with Merthiolate to poliomyelitis vaccine, as has been described for the stabilization of the vaccine solution, would affect the otherwise strong antiseptic activity of Merthiolate.² Serial dilutions of various samples of poliomyelitis vaccine containing Merthiolate 1:10,000 (0.000247 M) and varying EDTA concentrations were made in Pen assay broth, and 0.1 ml. of a 24-hour broth culture of *Staphylococcus aureus* was planted into 5 ml. amounts of these dilutions. Readings of turbidity were made after 24 hours' incubation at 37° C. Concentrations of EDTA from 1 to 100 moles per mole of Merthiolate did not affect the antistaphylococcal bacteriostatic action of Merthiolate. A second reading at 85 hours showed the same results as the 24-hour reading except in one tube which showed some growth in the presence of 1:1,600,000 Merthiolate.

Additional tests were done against certain fungi, with the same general result as follows: Dilutions of vaccines, both neutralized and nonneutralized, containing EDTA (0.00247 M), glycine (0.3 M), and EDTA and preserved with Merthiolate 1:10,000 were made up in medium 199; similar dilutions of Merthiolate 1:10,000, alone, were made up in medium 199. These serial doubling dilutions were planted with a heavy dose of spores of *Penicillium frequentis*, *Fusarium moniliforme*, *Mucor genevensis*, and *Aspergillus niger*. The tubes were left standing at room temperature for 10 days before readings were made. Spore controls in medium 199 grew out in 1 or 2 days. The fungistatic titers of the preparations containing Merthiolate and EDTA were the same as those without EDTA. These data agreed with our results on the antifungal action of Merthiolate published in 1934.⁹ It appears there is no suppression of the fungistatic or bacteriostatic activity of Merthiolate by EDTA.

Effect of Thiosalicylate Ion on Merthiolate.—Twenty-five parts per million of Merthiolate completely inhibited growth of a mixture of 13 yeasts in Sabouraud media for 14 days. The addition of 8×10^{-3} M thiosalicylate ion sufficiently reduced the fungistatic activity of Merthiolate at 100 parts per million for growth to appear after seven days. Although the rate of growth