FURTHER STUDIES ON THE STERILIZATION OF T.A.B.C. VACCINE

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IN a previous communication (Rainsford, 1937*a*) on research into the production of anti-typhoid vaccine of enhanced immunological quality, it was shown that it was possible to sterilize suspensions of *B. typhosus* without destroying the Vi antigen; the processes of sterilization employed were katadyn and electro-katadyn. These methods are, however, complicated and cannot be adapted easily to large-scale production of vaccine. In an endeavour to devise a more simple process, extended experiments have been carried out on the effect of the addition of solutions containing silver (as silver nitrate) and of mercury (as sodium merthiolate). Katadyn processes exert their effect by the oligo-dynamic action of silver, so in the present investigations silver nitrate was employed as its use gave promise of a method in which the amount of silver employed could be accurately controlled. Sodium merthiolate was chosen as it is soluble in normal saline solution, has a high bactericidal action, and is relatively harmless to human tissues when injected subcutaneously.

TECHNIQUE

Strain of B. typhosus *used*

All vaccines were prepared from the "V" strain Ty 2. Stock cultures of this organism, kept on inspissated egg medium, were maintained in their smooth, virulent, and O antibody resistant state by frequent mouse passage.

Cultivation of organisms

The suspensions employed were all prepared from 18 hr. old cultures grown at 37° C. in 32 oz. "UGB Bow Flats" (Rainsford, 1937*b*) on beef bouillon agar *p*H 7·4.

Counting of bacteria

In all these experiments the counting of bacteria was carried out by means of a Spekker Photoelectric Absorptiometer (Hilger). In this instrument a beam of light of constant intensity is arranged to pass through a glass vessel containing the solution or suspension under examination, and the amount of light that passes through the material is converted into an electrical effect by means of a photoelectric cell; this is recorded and measured by means of a galvanometer arranged in the circuit. It follows that if two galvanometer readings are taken, one with normal saline solution and one with a bacteria

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suspension, that the difference between the two will be related to the amount of light absorbed by the bacteria.

In the application of this instrument for counting bacteria, the galvanometer readings, in the first instance, require to be correlated to number of bacteria determined by other methods; galvanometer readings obtained in the calibration are plotted against number of bacteria, and the graphs obtained (one for each thickness of vessel) are available for permanent use. Thereafter a count can be made in less than a minute by reference of a galvanometer reading to the graph. The curves used in these experiments were constructed from the mean of figures obtained with various dilutions of a suspension of *B. typhosus* strain Ty 2 counted independently by three different workers each using both opacity and Helber slide methods. In the absorptiometer method of counting, therefore, the accuracy of any single count is according to the exactitude of the initial calibration, and the experimental error of the apparatus, but in a series of counts the error in calibration is constant and the exactitude of the figures in their relation to each other is affected only by the experimental error of the apparatus. This is so small that it can be disregarded. For this reason the employment of the absorptiometer method simplified and enhanced the accuracy of the experiments, especially those in which the minimum lethal dose of *B. typhosus* for mice had to be determined.

Experiments showing the bactericidal properties of silver nitrate in normal saline solution

Previous experiments with electro-katadyn had shown that although the silver imparted by this means was apparently insoluble in normal saline, it could exert a considerable bactericidal effect in such a medium. There was reason to believe then that silver precipitated as chloride from silver nitrate would also exert an antiseptic action in vaccine and prevent contamination. Experiments were made to ascertain whether this belief was well founded and if so the minimum amount of silver nitrate necessary.

Silver nitrate solution, 0·016% calculated as silver, was prepared with distilled water. The solution was added to an equal volume of 1·7% sodium chloride solution. A series of dilutions of this mixture was made using sterile 0·85% saline as the diluent. To prevent loss of water by evaporation and consequent alteration in the concentration of the salts present, 20·0 c.c. of each dilution was measured into a sterile 1 oz. screw-cap bottle. Each bottle was then contaminated with 0·1 c.c. of a 24 hr. old broth culture of *B. typhosus*; 20·0 c.c. of 0·85% sterile saline was treated in the same way to act as a control.

A similar experiment was made using a broth culture of staphylococcus as the contaminant. After being contaminated the caps of the bottles were screwed down tightly and the bottles allowed to stand at room temperature. Sterility tests were made daily by inoculating some of the solution from each

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bottle into broth tubes which were incubated for 1 week at 37° C. The results obtained (shown in Table I) show that normal saline to which silver nitrate has been added has considerable bactericidal properties.

PREPARATION OF VACCINES USING SILVER FOR STERILIZATION

Two methods involving the use of silver nitrate were examined.

Method 1. An aqueous solution of silver nitrate equivalent to 0·008% silver, was added to an equal volume of 1·7% saline solution. Equal volumes of this mixture and saline suspensions of *B. typhosus* were mixed together under sterile conditions in screw-cap bottles and stored in a refrigerator at 7° C.

Method 2. An aqueous solution of silver nitrate was prepared equivalent to 0·004% silver. Agar cultures suspended in distilled water were mixed with an equal volume of the silver solution, and the mixture was incubated at

Table I. *Bactericidal properties of silver nitrate in* 0·85% *saline*

Time of test in hours from moment of contamination	Contaminating organism	% of silver present					Saline control
		0·008	0·004	0·002	0·001	0·0005	
0	*B. typhosus*	+	+	+	+	+	+
0	*Staphylococcus*	+	+	+	+	+	+
24	*B. typhosus*	−	−	−	−	−	+
24	*Staphylococcus*	−	−	−	+	+	+
48	*B. typhosus*	−	−	−	−	−	+
48	*Staphylococcus*	−	−	−	+	+	+
72	*B. typhosus*	−	−	−	−	−	+
72	*Staphylococcus*	−	−	−	−	+	+

+ = Growth in test broth.
− = No growth in test broth after 1 week at 37° C.

37° C. for 4 hr. This was then decanted into a sterile screw-cap bottle containing sufficient sodium chloride to render the liquid isotonic. Having been well shaken, the suspension was placed in the refrigerator after samples had been taken for sterility tests.

In both methods the final concentration of the silver was therefore 0·002%. It was found that saline suspensions of *B. typhosus* could be sterilized by the first of these two methods in a period varying from 5 to 12 days.

The maximum weight of suspension subjected to this treatment was equivalent to 20×10^9 bacilli per c.c. The time necessary for sterilization to be effected was variable. With suspensions of over 10×10^9 bacilli per c.c. the average time was 11 days and the maximum 13 days. With suspensions of 5×10^9 per c.c. sterilization was usually effected in 8 days; on one occasion 5 days' contact was sufficient.

Using the second method, it was found that $7{\cdot}5 \times 10^9$ bacilli per c.c. could be satisfactorily killed in 4 hr. at 37° C., but more than this number could not be sterilized in this period. In the treatment by this method of suspensions not exceeding 20×10^9 per c.c. it was found that sterilization was completed

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after 4 hr. incubation at 37° C., the subsequent addition of sodium chloride, and then 3 days' storage in a refrigerator.

In carrying out the sterility tests in these experiments 0·5 c.c. of suspension was used for each test to ensure that adequate samples were utilized. This volume was distributed amongst 10 × 20 c.c. tubes of broth and incubated for 1 week at 37° C. No suspension was considered sterile until it had given negative results in all tubes in three separate tests made on consecutive days. This precaution was rendered necessary because it was found that after the first 2 days, suspensions subjected to the treatment described in method 1 contained very few viable bacilli but that following this initial period, the bactericidal effect of the silver became greatly diminished.

Some of the results obtained with these methods are shown in Table II.

Table II. *Sterilization of* B. typhosus *suspensions with silver nitrate*

Suspension	Suspending solution	No. of bacteria per c.c.	Result	Remarks
A.G.S. 1	Saline	5×10^9	Sterile after 5 days at 4° C.	—
A.G.S. 2	,,	10×10^9	Sterile after 8 days at 4° C.	—
A.G.S. 3	,,	20×10^9	Sterile after 13 days at 4° C.	—
A.G.S. 4	,,	13×10^9	Sterile after 12 days at 4° C.	—
A.G. 1	Water	2×10^9	Sterile after 4 hr. at 37° C.	Sodium chloride added after the first 4 hr. at 37° C.
A.G. 2	,,	7×10^9	Sterile after 4 hr. at 37° C.	
A.G. 3	,,	10×10^9	Sterile after 3 days at 4° C. following 4 hr. treatment at 37° C.	
A.G. 6	,,	13×10^9	Sterile after 3 days at 4° C. following 4 hr. treatment at 37° C.	

Preparation of vaccines using sodium merthiolate

Using saline suspensions of *B. typhosus*, two volumes of suspension were added to one volume of a solution of the following composition:

Sodium merthiolate ...	1·0 mg.
Sodium chloride	79·4 mg.
Sodium borate	14·0 mg.
Sterile distilled water ...	100 c.c.

(This solution is isotonic. Sodium borate was introduced at the suggestion of the manufacturers of the merthiolate to act as a buffer, as the compound tends to precipitate even in weak acid solutions.) The mixture after standing for 24 hr. at room temperature was tested for sterility. Suspensions of 10×10^9 bacilli per c.c. were satisfactorily sterilized in this way.

The effect of silver nitrate and of sodium merthiolate on the agglutinogens of "V" strains of *B. typhosus*

In the many investigations which have been made to discover a method of sterilization which would leave the Vi antigen content of a vaccine undiminished, a large number of processes and bactericidal substances have been examined. In these it has been observed that in nearly every case where

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a suspension after sterilization failed to stimulate the formation of Vi antibody in rabbits, the agglutination reactions obtained with the suspension showed an appreciable loss of resistance to O antibody. This property has, therefore, come to be regarded as an almost essential characteristic of a vaccine if it was to produce an immunity to infection with "V" strains of *B. typhosus*.

The agglutination reactions of both silver nitrate and of sodium merthiolate sterilized suspensions to pure Vi, O and H antisera are shown in Table III, and it will be seen that in this respect these scarcely differ from those obtained with live Vi containing bacilli.

Table III. *Showing the agglutination reactions of variously sterilized suspensions of strain* Ty 2

		Suspensions				
		Silver sterilized		Merthiolate sterilized	Living	
Serum	Dilution	A.G.S. 4	A.G. 6	M. 3	Ty 2	Ty 901
Pure Vi serum, titre 1/800	1/80	+ + +	+ + +	+ + +	+ + +	–
	1/160	+ + +	+ + +	+ + +	+ + +	–
	1/320	+ + +	+ + +	+ + +	+ + +	–
	1/640	+ +	+ +	+ +	+	–
	1/1280	+	+	+	(±)	–
	1/2560	–	–	–	–	–
Pure "O" serum, titre 1/10,000	1/40	+ +	+ +	+ +	+	+ + +
	1/80	+	+	+	(±)	+ + +
	1/160	(±)	+	(±)	(±)	+ + +
	1/320	–	(±)	–	–	+ +
	1/640	–	–	–	–	+ +
	1/1280	–	–	–	–	+ +
	1/2560	–	–	–	–	+ +
	1/5000	–	–	–	–	+
	1/10,000	–	–	–	–	(±)
H serum, Oxford, titre 1/250	1/250	+	+	+	+	+

THE IMMUNOGENIC PROPERTIES OF SUSPENSIONS STERILIZED WITH SILVER NITRATE AND OF THOSE STERILIZED WITH SODIUM MERTHIOLATE

To assess the immunogenic value of these new vaccines, their immunizing effect in rabbits was compared with that produced by live fully-virulent Vi containing bacilli and with a suspension sterilized by heating at 58° C. for $1\frac{1}{2}$ hr., followed by the addition of 0·5% phenol. This latter method of sterilization has been shown (Felix, 1934, 1935) to destroy all the Vi antibody stimulating properties of a vaccine.

Rabbits were immunized with suspensions sterilized by these different methods by giving three intravenous doses of 500, 1000 and 2000 million organisms respectively, with a 4 days' interval between each dose. Using live suspensions the procedure recommended by Felix (1934) of administering four intravenous doses of 100, 200, 200 and 300 million at 2-day intervals was adopted. This procedure occasionally made the rabbits extremely ill and they always lost considerable weight. Later it was discovered that if the animals received two preliminary doses of silver sterilized vaccine they could then be

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given further large doses of live cultures without apparent ill effect. Rabbit V 7 was immunized in this manner and received four intravenous doses of 500, 1000, 2000 and 2000 million organisms given at 4-day intervals; the first two doses consisted of silver sterilized vaccine and the last two of live virulent Vi containing bacilli.

One week after receiving the last injection each rabbit was bled from the heart, the serum was separated and after being passed through a Seitz E.K. filter was stored without preservative.

The Vi, O and H titres of these sera are shown in Table IV.

Table IV. *Showing the agglutinogenic properties of variously sterilized suspensions of* B. typhosus

Suspension used for immunization	Rabbit	Dose in millions	Titres of antibodies present in the sera: H	O	Vi (Watson)
Ty 2 sterilized by heating at 58° C. for 1½ hr. and subsequent addition of 0·5% phenol	P. 1	3500	10,000	5000	0
	P. 2	3500	10,000	5000	0
A.G. 1 = Ty 2 sterilized with silver nitrate	A.G. 1	3500	20,000	5000	1600
M. 3 = Ty 2 sterilized with 1/3000 merthiolate	M. 3	3500	20,000	5000	640
Living suspensions of Ty 2	V. 6	800	10,000	2500	1280
Silver sterilized, followed by living suspensions of Ty 2	V. 7	5500	10,000	2500	800

The protective power of these variously produced sera was compared and titrated by means of passive immunization experiments with mice. In these experiments only coloured mice of 25–30 g. weight were used, as it had been found that albino mice were more resistant to infection with "V" strains of *B. typhosus* than the coloured varieties. Mice in groups of ten were injected subcutaneously with the different sera. The maximum dose of serum injected was 1·0 c.c. and the minimum 0·25 c.c. In order to ensure accuracy when the latter dose was used, the serum was diluted with an equal volume of sterile saline, and 0·5 c.c. of the mixture was injected. 24 hr. after receiving the serum each mouse was injected intraperitoneally with a test dose of a living suspension of *B. typhosus* strain Ty 2 prepared from an 18 hr. agar culture and contained in a total volume of 0·5 c.c. normal saline. Test doses of 4 × M.L.D. and 8 × M.L.D. were employed.

For controls mice unprotected by serum were injected intraperitoneally with the test dose suspension suitably diluted to allow 1 × M.L.D. to be administered in a total volume of 0·5 c.c. The results of these experiments (shown in Table V) were observed over a period of 1 week and the number of survivors after this lapse of time noted.

These experiments would appear to indicate that in the application of either silver nitrate or of sodium merthiolate for the sterilization of suspensions of *B. typhosus* a process is available which is without immediate destructive effect on the Vi antigen or any of the antigens essential for a potent vaccine.

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However, in these experiments only freshly prepared vaccines were employed, i.e. no vaccine was more than 1 week old (dating from the time of completion of the sterility tests) when the first immunizing dose was given. The question,

Table V. *Showing the comparative protective value of variously produced anti-typhoid sera*

Suspension used for immunization	Serum	Dose of serum c.c.	Exp. 1. Results using 8 × M.L.D.	Exp. 2. Results using 8 × M.L.D.	Exp. 3. Results using 4 × M.L.D.
Ty 2 sterilized by heating at 58° C. for 1½ hr. and subsequent addition of 0·5% phenol	Rabbit P. 1	0·5	—	—	0/10
		0·25	—	—	0/10
	Rabbit P. 2	1·0	3/10	0/10	—
		0·5	1/10	1/10	—
		0·25	0/10	1/10	—
Ty 2 sterilized with silver nitrate	Rabbit A.G. 1	0·5	10/10	—	—
		0·25	10/10	—	—
Ty 2 sterilized with 1/3000 merthiolate	Rabbit M. 3	0·5	—	8/10	10/10
		0·25	—	7/10	10/10
Live suspensions of Ty 2	Rabbit V. 6	0·5	9/10	—	—
		0·25	8/10	—	—
Silver sterilized followed by live suspensions of Ty 2	Rabbit V. 7	0·5	—	9/10	9/10
		0·25	—	9/10	9/10
Controls receiving 1/3 of test dose suspension		0	—	—	0/5
Controls receiving 1/4 of test dose suspension		0	—	—	0/5
Controls receiving 1/5 of test dose suspension		0	0/5	—	—
Controls receiving 1/7 of test dose suspension		0	0/5	0/5	—
Controls receiving 1/8 of test dose suspension		0	0/5	0/5	—

Exp. 1. Test dose suspensions $=560 \times 10^6$ of Ty 2. $1 \times$ M.L.D. $=70 \times 10^6$.
Exp. 2. Test dose suspensions $=640 \times 10^6$ of Ty 2. $1 \times$ M.L.D. $=80 \times 10^6$.
Exp. 3. Test dose suspensions $=320 \times 10^6$ of Ty 2. $1 \times$ M.L.D. $=80 \times 10^6$.
Denominator = no. mice tested.
Numerator = no. of survivors after 1 week.

therefore, as to whether there would be any advantage in employing one or other of these new vaccines in place of the present heat-killed and phenolized vaccine must depend upon the permanence of their antigenic content under various conditions of storage.

Investigations into the antigenic stability of vaccines

The subjects of these investigations were the following vaccines.

Vaccine K. 2. This suspension was sterilized by the katadyn process. It originally consisted of a suspension of 15×10^9 organisms per c.c. This was diluted one-third with sterile katadyn treated normal saline solution and after being bottled was stored in the refrigerator at 7° C.

Vaccine A.G.S. 4. The suspension A.G.S. 4 (see Table I) was diluted down to an opacity equal to 2500×10^6 organisms per c.c. and the total volume of vaccine thus obtained was divided into two equal portions. To one portion sufficient silver nitrate was added to allow a final concentration of 0·001% silver to remain in the vaccine, and in the other double this amount. These two vaccines were bottled off separately in amber vaccine bottles and some of each was stored in the dark at room temperature and others at 7° C.

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Vaccine A.G. 6. The suspension A.G. 6 (*vide supra*, Table I) was diluted down to an opacity equivalent to 5000×10^6 organisms per c.c. with sterile 0·85% saline containing 0·002% silver. After being bottled it was stored at 7° C.

Vaccine M. 3. A suspension of *B. typhosus* $10{,}000 \times 10^6$ organisms per c.c. was sterilized with a concentration of 1/3000 sodium merthiolate. This was then diluted one-half with normal sterile saline, and stored in the refrigerator at 7° C.

The vaccines A.G.S. 4, A.G. 6 and M. 3 were examined after a period of 6 months, and K. 2 after 18 months' storage.

Before proceeding to an antigenic analysis of these vaccines, their sterility was tested. The tests showed that the vaccines A.G. 6, K. 2 and M. 3 were sterile but that approximately 30% of the bottles containing the vaccine A.G.S. 4 were contaminated with a mould. This contamination was probably introduced with the silver nitrate when it was added to the vaccine after the original suspension was diluted with sterile saline to its required opacity. This was the only vaccine in which silver in an unsterilized form was used; it had been found that sterilization in the autoclave of silver in solution whether in saline or in water had no detrimental effect on its bactericidal action. This was true of both forms of silver experimented with, i.e. katadyn and silver nitrate. Although to date no vaccine prepared with sterilized silver solution has become contaminated, further investigations into the problem of preventing the growth of moulds in silver preserved vaccines are being made as it is apparent that the concentration of silver used in these experiments is insufficient alone to prevent this kind of secondary growth.

The immunogenic value of vaccines after storage

The procedure for ascertaining the immunological effectiveness of these vaccines after they had been stored was similar in all details to that adopted in the earlier experiments described in this paper. Rabbits were immunized with the various vaccines, and bled; their sera was then examined by *in vitro* tests and by means of passive immunization experiments. In these experiments, as in the earlier investigations, the sera from animals immunized with live fully virulent cultures, and also those from animals immunized with freshly prepared heat-killed phenolized vaccines of Vi containing bacilli, were used for comparison.

Whilst the fallacy of drawing conclusions from so small a number of experiments is of course appreciated, the following analyses of the results shown in Tables VI and VII give ground for the belief that the presence of Vi agglutinins in any of these sera is indicative of true Vi antibody. Table VII shows that with the exception of the two sera K. 2 and V. 8 the degree of protection afforded by any serum was proportional to its Vi antibody content as demonstrated by *in vitro* tests (Table VI).

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Table VI. *Showing the effect of storage under various conditions on the agglutinogenic properties of silver preserved and of merthiolate preserved vaccines*

Suspension used for immunization		Preservative	No. of organisms per c.c.	Storage temp. °C.	Age of vaccine months	Rabbit serum	Dose in millions	Titres of antibodies present in the sera: Vi (Watson)	O	H
Ty 2 sterilized with silver nitrate by (method 1):	A.G.S. 4	Silver 0·001%	2500×10^6	7	6	J. 1	3500	320	5000	5,000
	A.G.S. 4	Silver 0·001%	2500×10^6	Room	6	R. 1	3500	80	5000	5,000
	A.G.S. 4	Silver 0·002%	2500×10^6	7	6	J. 2	3500	160	2500	5,000
	A.G.S. 4	Silver 0·002%	2500×10^6	Room	6	R. 2	3500	60	1280	10,000
Ty 2 sterilized with silver nitrate (method 2)	A.G. 6	Silver 0·002%	5000×10^6	7	6	A.G. 6	3500	320	5000	5,000
Ty 2 sterilized with katadyn	K. 2	Silver 0·002% (approx.)	5000×10^6	7	18	K. 2	3500	160	5000	5,000
Ty 2 sterilized with merthiolate	M. 3	Merthiolate 1/6000	5000×10^6	7	6	M. 31	3500	0	2500	1280
Freshly prepared suspensions of Ty 2 sterilized by heating at 58° C. for 1½ hr. followed by the addition of 0·5% phenol		Phenol 0·5%	1000×10^6	—	—	P. 1	3500	0	5000	10,000
						P. 2	3500	0	5000	10,000
						P. 4	3500	0	5000	10,000
Living suspensions of Ty 2 preceded by freshly prepared suspensions sterilized with silver nitrate		None	—	—	—	V. 7	5500	800	2500	10,000
Living suspensions of Ty 2		None	—	—	—	V. 8	800	1280	1280	2,500

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The serum V. 8 afforded less and K. 2 slightly more protection than was to be expected if only their Vi titres are considered. The anomalous nature of the results obtained with these two sera can be explained to some extent by the recent work of Grasset & Lewin (1937) who have shown that the maximum

Table VII. *Showing the comparative protective value of sera produced by immunization with stored vaccines*

Suspension used for immunization	Rabbit serum	Dose of serum c.c.	Exp. 1. Result using 8 × M.L.D.	Exp. 2. Result using 4 × M.L.D.	Exp. 3. Result using 4 × M.L.D.
Freshly prepared suspensions of Ty 2 sterilized by heating at 58° C. for 1½ hr., followed by the addition of 0·5 % phenol	P. 1	0·25	—	0/10	—
	P. 1	0·50	—	0/10	—
	P. 2	0·25	1/10	—	—
	P. 2	0·50	1/10	—	—
	P. 2	1·00	0/10	—	—
	P. 4	0·25	—	—	0/10
	P. 4	0·50	—	—	0/10
Suspension K. 2 = Ty 2 sterilized with katadyn and stored for 18 months at 7° C.	K. 2	0·25	3/10	6/10	—
	K. 2	0·50	2/10	7/10	—
Suspension A.G.S. 4 = Ty 2 sterilized with silver nitrate (method 1) and stored at 7° C. for 6 months and preserved with 0·001 % silver	J. 1	0·25	—	—	8/10
	J. 1	0·50	—	—	9/10
Suspension A.G.S. 4 preserved with 0·002 % silver and stored for 6 months at 7° C.	J. 2	0·25	—	—	3/10
	J. 2	0·50	—	—	7/10
Suspension A.G. 6 sterilized with silver nitrate (method 2) and preserved with 0·002 % silver and stored for 6 months at 7° C.	A.G. 6	0·25	—	—	9/10
	A.G. 6	0·50	—	—	9/10
M. 3 = Ty 2 sterilized with merthiolate and preserved with 1/6000 merthiolate and stored for 6 months at 7° C.	M. 31	0·25	—	—	0/10
	M. 31	0·50	—	—	1/10
Living suspensions of Ty 2 preceded by freshly prepared suspensions, sterilized with silver nitrate	V. 7	0·25	9/10	9/10	—
	V. 7	0·50	9/10	8/10	—
Living suspensions of Ty 2	V. 8	0·25	—	—	9/10
	V. 8	0·50	—	—	8/10
Controls receiving 1/8 test dose suspension		0	0/5	—	—
Controls receiving 1/7 test dose suspension		0	0/5	—	—
Controls receiving 1/4 test dose suspension		0	—	0/5	0/5
Controls receiving 1/3 test dose suspension		0	—	0/5	0/5

Exp. 1. Test dose suspension = 560×10^6. 1 × M.L.D. = 70×10^6.
Exp. 2, 3. Test dose suspension = 320×10^6. 1 × M.L.D. = 80×10^6.
Denominator = no. mice tested.
Numerator = no. of survivors after 1 week.

protection against infection with V strains of *B. typhosus* can be obtained only with a serum containing both Vi and O antibody. It has been seen (Table VI) that although the Vi titre of serum V. 8 was high, the O titre was comparatively low, and the deficiency of the latter antibody probably explains why its protective qualities were less than might have been anticipated. This

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theory is supported by the results of the experiments made with the sera K. 2 and J. 2; both of these had the same Vi titre but the serum K. 2 contained twice as much O antibody as J. 2, and afforded the better protection.

These experiments, therefore, demonstrate that a vaccine preserved with 1 : 6000 sodium merthiolate failed after storage for only 6 months at 7° C. to stimulate any Vi antibody response in the rabbit. This may not necessarily be due to a directly destructive effect by the merthiolate on the Vi antigen, for Felix (1937) found that alcohol-killed suspensions stored for the same period but without any preservative added were equally ineffective. It is more probable, therefore, that this loss of Vi antigen is due to an inherent instability of this substance. On the other hand the experiments show that silver, while it does not entirely prevent the loss of this antigen, appears to have some effect by which the rate of loss is greatly retarded. However, this effect is only obtained when the vaccine is stored at a low temperature. It would also appear that the weight of silver which can be added to a vaccine to preserve the Vi antigen is limited, and must be in proportion to the weight of bacteria per c.c. in the vaccine, for a vaccine of 2500×10^6 organisms per c.c. with 0·002 % silver added, produced only half as much Vi antibody as a vaccine with the same count but containing 0·001 % silver; further a vaccine with a count of 5000×10^6 organisms per c.c. and with 0·002 % of silver, produced Vi antibody in amount equal to that produced by a vaccine with half the count and half the amount of silver, i.e. 0·001 %. These three vaccines had all been stored under the same conditions for 6 months.

It may be remarked that the author in order to test whether any injury might follow the injection of vaccines containing silver, injected himself subcutaneously with 1·0 c.c. of a solution of 0·85 % saline containing 0·002 % of silver (added in the form of silver nitrate); the injection caused no discomfort and, except for an area of half an inch immediately surrounding the site which was pinkened for 24 hr., there was no coloration of the skin.

Summary

1. Silver nitrate or sodium merthiolate can be used for the sterilization of suspensions of *B. typhosus* and neither have any immediate destructive effect on the Vi, O or H antigens.

2. Two methods of employing silver nitrate as a sterilizing agent are described.

3. Physiological saline solution with silver nitrate added is bactericidal for *B. typhosus* and the staphylococcus but has no inhibitory action on the growth of moulds.

4. Vaccines sterilized and preserved with silver nitrate after storage for 6 months at 7° C. were found to have retained their Vi antibody stimulating properties.

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5. A vaccine sterilized and preserved with "katadyn" after storage for 18 months at 7° C. still contained some Vi antigen and was superior as an immunogenic agent to a freshly prepared heat-killed-phenolized vaccine.

6. It would appear that the stability of Vi antigen is increased by the addition of silver within certain limits, but this effect is only obtained if low temperature storage conditions are maintained.

7. A vaccine sterilized and preserved with sodium merthiolate after 6 months' storage at 7° C. failed to stimulate any Vi antibody in the rabbit.

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