THE PRESERVATION OF Vi ANTIGEN IN T.A.B.C. VACCINE

WITH A NOTE ON COMBINED ACTIVE IMMUNIZATION WITH T.A.B.C. VACCINE IN TETANUS FORMOL-TOXOID

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(With 2 Figures in the Text)

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I. Introduction

The question has been raised, following the discovery by Felix (1934), of the Vi antigen of *Bact. typhosum*, and of the important role played by its antibody in the development of an immunity against infection with this organism, as to whether the immunological qualities of T.A.B.C. vaccine could be improved.

The method of preparing antityphoid vaccine, almost universally employed, namely, sterilization of *Bact. typhosum* suspensions by heat, and preservation with 0·5 % phenol, would appear to destroy to a great extent the immunogenic property of Vi antigen. Felix (1934) has shown that the sera of rabbits immunized with suspensions of V strains killed by heating at 58° C. or by 0·5 %

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phenol (Felix & Bhatnagar, 1935) contain no Vi antibody or only negligible amounts of it. That Vi antigen is not completely destroyed by either of these methods is shown by the fact that mice immunized with heat-killed phenolized vaccine develop an active immunity against experimental infection with fully virulent organisms (Perry, Findlay & Bensted, 1934).

Man, however, would appear to react in a manner more closely resembling that of the rabbit than that of the mouse, for Felix, Rainsford & Stokes (1941) found that following immunization with heat-killed phenolized vaccine only 5–7 % of men developed Vi agglutinins in their sera.

It is reasonable therefore to assume that suspensions of *Bact. typhosum* killed by any method which has no inhibitory action on their Vi antibody-stimulating properties in the rabbit, e.g. the addition of 75 % alcohol, will have an enhanced immunological quality for man. This assumption has to some extent been confirmed by Felix *et al.* (1941), who found that in a group of men, none of whom had been immunized previously against typhoid or had suffered from enteric fever, 40 % developed Vi agglutinins in their sera after immunization with an alcohol-killed and preserved T.A.B.C. vaccine.

Rainsford (1937, 1939) has shown that suspensious of *Bact. typhosum* can be sterilized by silver in the form of Katadyn or silver nitrate without destroying the Vi antigen. Both these processes, however, have serious defects. Silver as a sterilizing agent is slow in action and as a preservative it does not prevent the growth of moulds; further, suspensions sterilized and preserved with silver, while they retain their potency to stimulate the production of Vi antibody in the rabbit for as long as 18 months when stored at 7° C., rapidly lose this property if stored at room temperature, i.e. 23–25° C.

It has been shown by Felix & Pitt (1936) that suspensions of *Bact. typhosum* in physiological saline can be sterilized by the addition of 75 % by volume of absolute alcohol, and more recently Felix (1941) has shown that suspensions treated in this manner when preserved with 25 % alcohol in physiological saline will, after several months' storage at 0–2° C., stimulate the formation of Vi antibody in both rabbits and man. No information is available regarding the behaviour of this vaccine when stored at 23–25° C.

The entire supply of T.A.B.C. vaccine for the Royal Navy is prepared at the Royal Naval Medical School, Clevedon. From here stocks are despatched to distributing centres all over the world, and a consigment may be several months in transit and again in store for a considerable period, as large amounts of vaccine must be ready for immediate issue. Under present conditions it is not practicable to guarantee that the vaccine will be kept at ice-box temperature throughout the whole of this period, and all that can be assured is that it will be stored in a cool place. It will be seen, therefore, that to provide the Royal Navy with an improved T.A.B.C. vaccine presents two separate problems, the evolution of a process of sterilization which will leave the Vi and O antigens unaltered, and a method which will preserve these antigens from deterioration when the vaccine is stored for several months at 23–25° C.

For the immunization of the fighting forces it has been advocated as a war measure that combined active immunization against both tetanus and typhoid by means of T.A.B.C.-vaccine-tetanus-formol-toxoid mixture should be adopted. While this method of immunization has been shown by various workers (Ramon & Zoeller, 1927; Maclean & Holt, 1941) to be beneficial in respect of the degree of active immunity produced against tetanus, it is by no means certain, in the light of recent knowledge, that it is also true for typhoid and its allied infections.

The present paper reports an experimental investigation into these various problems.

II. METHODS AND TECHNIQUE

For details of methods and technique reference may be made to two earlier papers (Rainsford, 1937, 1939).

(*a*) *Strain of* Bact. typhosum *employed*

Throughout the experiments the various vaccines examined were all prepared with the V strain Ty 2; it was maintained fully virulent and O resistant by methods previously described.

(*b*) *Immunization of rabbits*

The immunogenic properties of the various vaccines under investigation were tested by inoculation into rabbits. The method of immunization employed was the same in all details as that described in the earlier papers. No rabbit was used for test whose serum prior to immunization had a Vi or H agglutinin titre of 1/10 or higher, or an O titre of more than 1/40. (Only one rabbit of sixty tested had to be rejected because of the presence of Vi agglutinins in its serum.)

(*c*) *The estimation in vitro of the antibodies in the rabbit sera*

The O and H titre of each serum was titrated by the agglutination test using a suspension of the strain H 901 for the H, and O 901 for the O antibody. These tests were carried out in accordance with the technique of Felix & Gardner (1937).

The estimation of the Vi titre was made by titrating each serum with a formolized suspension of *Salmonella typhi* Watson after the O and H agglutinins had been removed by absorption with a very heavy suspension of the W strain Ty H 901. With some sera of exceptionally high anti-O titre, it was found that absorption at a dilution 1/20 was insufficient to remove all the O antibody. Before the Vi titre was finally estimated, and where the Vi titre was such that it allowed of further dilution of the serum without being diluted out, the serum was reabsorbed until all the agglutinins other than Vi had been removed. Those sera of high O but low Vi titre were absorbed to a dilution of 1/20, and this usually reduced the O agglutinin content to the extent that this antibody, in the absence of Vi antibody, would not have produced any agglutination of

Watson suspension, i.e. the O titre after absorption at 1/20 was less than 1/500. The Vi titre was then titrated. However, as an additional control, whenever a titration of Vi antibody was made the O resistance of the Watson suspension used was checked against a pure O serum, which had an O titre of not less than 1/10,000, this test being run in parallel with that of the Vi titration. No test was considered valid if the Watson suspension agglutinated with the O serum to more than 1/500th of its titre, i.e. in a dilution of 1/40 of a serum with an O titre of 1/10,000.

To confirm these results each serum was titrated unabsorbed against a formolized suspension of *S. ballerup* (Kauffman & Møller, 1940). It was found that *S. ballerup* suspension agglutinated with Vi antibody to half the titre obtained with Watson suspension. By standardizing the Watson and *S. ballerup* suspensions against a provisional standard Vi serum a very close agreement was obtained between these two methods of titrating Vi antibody. Before adopting this procedure a large number of normal rabbit sera were tested against whole and alcohol-treated *S. ballerup* suspensions. None showed the presence of H antibody for *S. ballerup*, and none had a higher titre than 1/40 for the alcohol-treated suspension. Of all the sera tested against the whole suspension, only one showed a trace of agglutination at a dilution of 1/10, the lowest dilution tested. This serum was from the rabbit which had to be rejected.

(*d*) *The passive protection test in the mouse*

The functional efficacy and protective value of the Vi antibody in the variously produced immune sera was titrated by means of passive protection experiments with mice. The technique used was the same as that described earlier with the exception that as a unit of dosage of the test dose suspension, the average lethal dose (A.L.D.) in the place of the minimum lethal dose (M.L.D.) was employed. The A.L.D. was that dose which would kill at least 50 % of normal mice in 48 hr. The adoption of this method is responsible for what appears to be a discrepancy in Exps. I and III shown in Table 9, for it will be seen that of the ten controls that received 1 × A.L.D. only one survived for 1 week, whereas in Exp. II four survived. In Exp. II, however, all the controls died within 48 hr. of being injected, but in Exps. I and III, three of ten that received 1 × A.L.D. died after a longer period.

III. The behaviour of vaccines sterilized and preserved with merthiolate and colloidal silver when stored at temperatures of 0–2 and 23–25° C.

Previous research (Rainsford, 1939) had shown that saline suspensions of *Bact. typhosum* could be killed rapidly with *sodium merthiolate* without any immediate destructive effect on the Vi antigen. However, vaccines prepared from suspensions killed by this method when preserved with 1/6000 merthiolate showed a rapid loss of Vi potency even when stored at ice-box temperature.

An examination of these vaccines after 6 months' storage at 7° C. showed that their *p*H concentration had fallen, and it was thought that the development of this acid reaction was probably the cause for the loss of Vi antigen. The merthiolate solution employed in these earlier experiments was buffered with sodium borate, and it was therefore possible that sodium borate, in addition to its apparent failure to buffer, might itself have had some deleterious effect. For these reasons experiments were commenced with a more alkaline solution of merthiolate than that previously used, namely, *Merthiolate Solution* no. 45 (Eli, Lilly and Co.). This is an isotonic solution with a *p*H of 10·0 and contains 0·1 % sodium ethyl mercuric thiosalicylate, and 0·1 % monoethanolamine. Further, in view of the partial success already obtained with silver as preservative of Vi antigen, it was decided to examine the possibility of employing this metal together with merthiolate in a vaccine. It was thought that by utilizing the silver in a colloidal state the complications arising from the incompatibility of mercurial salts with those of other heavy metals might be avoided.

A specially prepared and phenol-free *Collosol Silver Solution* containing 0·05 % silver was obtained from Crookes Laboratories, Ltd. The bacteriocidal properties of this colloidal suspension of silver were ascertained by the same technique as that used previously for silver nitrate (Rainsford, 1939), and it was found that it required double the amount by weight of silver in this colloidal form to produce the same bacteriocidal effect in physiological saline as when the silver was added as silver nitrate, i.e. 0·004 % by weight of silver in colloidal suspension had approximately the same effect as 0·002 % as silver nitrate.

Using these two solutions, five different vaccines were prepared.

Preparation of vaccines using merthiolate sterilized suspensions

Vaccine 1. Cultures incubated at 37° C. for 24 hr. on beef bouillon-agar, were washed off and suspended in physiological saline. An equal volume of *Merthiolate Solution* no. 45 was added to the suspension and the mixture placed in a refrigerator at 0–2° C. Sterility tests were made daily by distributing 0·3 c.c. of the suspension into two bottles each containing 150 c.c. of beef broth to which had been added 0·1 % sodium sulphide, and these were incubated for 1 week at 37° C. The suspension was not considered to be sterile until it gave negative results in tests made on three consecutive days. Usually it was found that suspensions of an opacity equivalent to 40×10^9 organisms per c.c. were sterilized in this way in 48–72 hr. When sterile a portion of the suspension was centrifuged at high speed until the bacterial cells had deposited; the supernatant fluid was discarded, and the deposited bacteria resuspended in merthiolate solution 1/10 in physiological saline, thus leaving a final concentration of 1/10,000 merthiolate in the suspension. It was further diluted with 1/10,000 merthiolate in physiological saline to an opacity equivalent to 5000×10^6 organisms per c.c.

Vaccine 2. Prepared as described for vaccine 1, but the final concentration of merthiolate was 1/5000.

Vaccine 3. Another portion of the merthiolate sterilized suspension was treated in a similar manner to vaccine 1, with the exception that the centrifuged deposit was resuspended 1/10,000 merthiolate in physiological saline plus sufficient *Collosol Silver Solution* to give a final concentration of 0·008 % of silver in the final product.

Preparation of vaccines using colloidal silver sterilized suspensions

Vaccine 4. Sufficient *Collosol Silver Solution* was added to a suspension of *Bact. typhosum* in physiological saline to give a final concentration of 0·008 % silver in the mixture and placed in a refrigerator at 0–2° C. Sterility tests were made daily by the technique already described. It was found that providing the suspension was of an opacity not greater than $15{,}000 \times 10^6$ per c.c., sterility was generally attained within a week. When sterile the opacity of this suspension was adjusted to 5000×10^6 per c.c. by diluting it with saline and sufficient *Collosol Silver Solution* to maintain a final concentration of 0·008 % silver in the vaccine.

Vaccine 5. This was prepared in the same manner as vaccine 4, except that the final concentration of silver allowed was only 0·004 %.

The vaccines when prepared were bottled in 50 c.c. rubber-capped amber bottles. Sterility tests in accordance with the Therapeutic Substances Act were made, broth containing 0·1 % sodium sulphide again being used for testing for the presence of aerobes. If the sterility tests were satisfactory, samples of each vaccine were stored, some at 0–2 and others at 23–25° C.

The agglutination reactions of these vaccines were tested against pure Vi and O sera, and the freshly prepared vaccines all gave reactions which differed little from those obtained with a live suspension of Ty 2. It will be seen, however, in Table 1 that although the vaccines after being stored for 1 year at 0–2° C. showed only a slight degree of increased sensitivity to both O and Vi antibody, they all, after 5 months' storage at room temperature showed a complete loss of resistance to O antibody, and a failure to agglutinate in the presence of Vi antibody. It was therefore concluded that while a small loss of Vi antigen had occurred in the vaccines stored at 0–2° C. for 1 year, there was almost a complete loss of this antigen from the vaccines stored for 5 months at room temperature.

The response produced in rabbits following immunization with these vaccines is shown in Table 2. It will be seen from this table that the conclusions drawn from the results obtained with the agglutination tests were confirmed, for all the rabbits immunized with vaccines which had been stored for 5 months at room temperature showed a very poor Vi antibody response, and with the exception of the rabbit L/1, all the animals that received the vaccines which had been stored at 0–2° C. gave a very good response in this respect.

Table 1. *Showing the agglutination reactions of vaccines prepared and preserved with merthiolate and colloidal silver in physiological saline after being stored at* 0–2° C. *and at* 23–25° C.

Vaccine ...	Ty 2 in physiological saline, sterilized with 0·008 % colloidal silver						Ty 2 in physiological saline, sterilized with 1/2,000 merthiolate						Ty live
Preservative ...	Colloidal silver, 0·008 %			Colloidal silver, 0·004 %			Merthiolate, 1/10,000			Merthiolate, 1/10,000 + colloidal silver, 0·004 %			Nil
Storage temp. °C. ...	0–2		23–25	0–2		23–25	0–2		23–25	0–2		23–25	
Months stored ...	5	12	5	5	12	5	5	12	5	5	12	5	
Pure anti-O serum dilutions, titre for O 901 = 1/10,000 — 1/20	±	−	+++	±	+	+	−	−	+	−	−	+++	−
1/40	±	−	+++	±	+	+	−	±	++	−	±	+++	−
1/80	−	±	+++	−	±	++	−	+	+++	±	+	+++	−
1/160	−	+	+++	−	±	+++	±	±	+++	+	+	+++	−
1/320	−	+	+++	−	±	+++	±	±	++	±	±	++	−
1/640	−	±	++	−	±	+++	±	±	++	±	±	++	−
1/1,280	−	±	++	−	−	++	±	−	++	−	±	++	−
1/2,560	−	±	+	−	−	+	−	−	±	−	±	+	−
1/5,000	−	−	±	−	−	±	−	−	±	−	−	±	−
1/10,000	−	−	−	−	−	−	−	−	−	−	−	−	−
Pure Vi serum dilutions, titre for Watson = 1/1,280 — 1/100	+++	++	±	++	++	±	++	++	+	+++	+++	±	+++
1/200	++	++	−	++	++	−	++	++	±	++	+++	±	++
1/400	++	++	−	++	++	−	++	++	±	++	++	−	++
1/800	+	+	−	+	+	−	+	+	−	++	++	−	±
1/1,600	−	−	−	−	−	−	−	±	−	±	+	−	−

Explanation of symbols

Symbol	Reading of deposit on bottom of the tube
1. −	Deposit on bottom of tube shows central area of unagglutinated cells. Supernatant milky and homogeneous.
2. ±	A central area of unagglutinated cells surrounded by a few agglutinated cells, which are seen only by the aid of a 2 in. hand lens. Supernatant as in 1.
3. ±	The central area of unagglutinated cells is smaller than that in a negative tube, and is surrounded by an area of agglutinated cells which are visible to the naked eye. Supernatant as in 1.
4. +	Deposit shows no central area of unagglutinated cells for the bottom of the tube is completely and evenly covered by a deposit of agglutinated cells. Supernatant is milky but contains clumps of agglutinated organisms just visible to the naked eye.
5. ++	As in 4 except that the supernatant is partly cleared and contains clumps of agglutinated cells easily visible to the naked eye.
6. +++	As in 5 except that the supernatant is completely cleared.

The agglutination tests were carried out in a total volume of 1·0 c.c., in 2 × ½ in. round bottomed tubes. These were incubated at 37° C. for 2 hr. and readings were made after they had been allowed to stand for 22 hr. at room temperature. The degree of agglutination present in each tube was estimated by examining both the deposit on the bottom of the tube, and the supernatant. A key to the symbols denoting the degree of agglutination seen in each tube is shown above.

By means of passive protection tests with mice, it was found (see Table 9) that these Vi agglutinins represented true Vi antibody, fully protective in quality.

The response obtained with rabbit L/1 to vaccine 2, suggests that a high concentration of merthiolate in a vaccine may have some detrimental effect, and the earlier experiments (Rainsford, 1939), in which it was found that a rapid deterioration of Vi antigen occurred at 7° C. in a vaccine preserved with 1/6000 merthiolate, lend support to such a conclusion.

Table 2. *Showing the effect of storage at* 23–25° C. *and at* 0–2° C. *on the agglutinogenic properties of vaccines prepared with physiological saline, compared with one prepared with* 32 % *saline solution*

Suspension used for immunization						Titre		
Method of sterilization	Preservative	Storage temp. °C.	Age of vaccine in months	Rabbit	Dose in millions	H	O	Vi
Colloidal silver 0·008 % in physiological saline solution	Colloidal silver 0·004 %	0– 2	7	D/B	3500	10·000	10,000	1280
		23–25	5	3/B	,,	5,000	10,000	<20
	Colloidal silver 0·008 %	0– 2	7	D/I	,,	2,500	2,500	640
		0– 2	12	L/3	,,	20,000	20,000	500
Merthiolate 1/2,000 in physiological saline solution	Merthiolate 1/10,000	0– 2	12	H/2	,,	20,000	10,000	1280
		23–25	5	W/3	,,	20,000	10,000	<20
	Merthiolate 1/5,000	0– 2	12	L/1	,,	10,000	10,000	100
	Merthiolate 1/10,000 + colloidal silver 0·008 %	0– 2	6	D/D	,,	5,000	5,000	1280
		0– 2	6	D/H	,,	5,000	5,000	1000
		0– 2	12	L/2	,,	20,000	20,000	500
		23–25	5	2/B	,,	5,000	5,000	80
Merthiolate 1/2,000 in 32 % saline solution	Merthiolate 1/10,000 in 32 % saline solution	23–25	6	W/7	,,	10,000	10,000	1400
			7	N/7	,,	20,000	40,000	1500

It would seem, nevertheless, that a concentration of 1/10,000 merthiolate is as good a preservative for typhoid vaccine as colloidal silver, which when employed in the manner described above, appears to have no harmful effect on either the Vi or O antigen, but silver alone will not prevent the growth of moulds, and in this respect merthiolate is to be preferred. It must be concluded, however, that neither silver nor merthiolate act as a satisfactory preservative of Vi antigen, for neither will prevent the loss of this antigen from a vaccine when stored at room temperature.

IV. The agglutination reactions of merthiolate sterilized and preserved suspensions following exposure at temperatures of 25, 37 and 60° C.

The marked difference between the immunogenic potency of vaccines stored at 0–2° C. and those stored at room temperature demonstrated the pronounced influence of temperature on the rate of loss of Vi antigen. It was thought that a comparison of the persistence of Vi antigen at temperatures of 25, 37 and 60° C. might afford information of value. An intensive study was therefore made of the agglutination reactions to pure O and Vi sera of a sterile suspension in physiological saline stored at these various temperatures. The

suspension used was one prepared, sterilized, and preserved in accordance with the technique described for the preparation of vaccine 1. The results obtained were similar to those shown in Tables 4 and 6, and it will be seen that when stored at 25° C. there is no evidence of any loss of Vi antigen after 2 months' storage at this temperature, but after 4 months' storage the reactions are those of a complete loss of this antigen. At 37° C. the deterioration appears to set in at once and is complete in 3–5 days, and at 60° C. it is complete in 4 hr. Tests were made with heavier suspensions than 5000×10^6 per c.c. to ascertain whether the concentration of organisms per c.c. had any influence on the result, and, in addition, the effect if any of bottling in vacuo was examined. It was found that a very heavy suspension of 80×10^9 per c.c. did appear to deteriorate a little more slowly at 25° C. than that of 5000×10^6 per c.c., but at temperatures of 37 and 60° C. the difference between the reactions of these two suspensions was insignificant, nor did storing in vacuo influence the result in any way. Thus it was concluded that 4 days' storage at 37° C. had approximately the same effect as 4 months at room temperature or 4 hr. at 60° C.

It appeared therefore that a study of the reactions of a vaccine exposed to a temperature of 37° C. would afford a rapid test for predicting its behaviour at room temperature, and that a vaccine in which the Vi antigen was rendered sufficiently heat-stable to withstand an exposure of 20–50 days at 37° C., would in all probability withstand many months of exposure to a temperature of 23–25° C.

V. THE IMMUNOGENIC PROPERTIES OF ACETONE-DRIED VACCINES AFTER BEING STORED AT 37 AND 25° C.

Henderson, Amies & Steabben (1940) have found that acetone-dried and killed bacilli of V strains of *Bact. typhosum* are capable of stimulating the Vi antibody in the horse at least as readily as the living organisms. An experiment was made to compare the keeping qualities of such dried vaccines with those of physiological saline suspensions preserved with 1/10,000 merthiolate. Using the same technique as that described earlier, a quantity of vaccines 1 and 3 were prepared in the first instance. These vaccines were centrifuged until all the cells had deposited, the supernatant fluid discarded, the deposit washed three times in acetone and then dried in vacuo. The dried deposit was stored in screw-capped bottles in the incubator at 37° C., together with a bottle of undried vaccine 1 which had been prepared from the same suspension. A bottle of dried vaccine 3 was placed in the refrigerator and retained at 0–2° C. to act as a control.

Rabbits were immunized with these three vaccines after they had been exposed for various periods of time to these temperature conditions. The dried vaccines prior to being injected were resuspended in 1/10,000 merthiolate in physiological saline, and during the 8 days interval which elapsed between the administration of the first and last dose to each rabbit, the suspensions were

kept in the refrigerator at 0–2° C. The result of this experiment is shown in Table 3.

All the vaccines that had been stored in the dry state at 37° C., even after 3 months' exposure to this temperature, produced a good Vi antibody response, equally as good as that of the vaccine stored at 0–2° C., while the physiological saline suspensions, after only 8 days' exposure at 37° C. failed in this respect.

It has been possible to test the immunogenic properties of merthiolate-killed acetone-dried bacilli after 1 year's storage at 23–25° C. The response obtained in rabbit N/6 following immunization with this vaccine is also shown in Table 3, and the protective power of this serum as titrated by passive immunization experiments in mice in Table 9. From these results it is evident that the stability of Vi antigen when in the dried state is of a very high order.

VI. The agglutination reactions of a merthiolate-killed and preserved suspension in 32 % saline solution after being stored at 37 and 25° C.

The immunogenic reactions obtained with dried vaccines and the reported successful preservation of Vi antigen with alcohol (Felix, 1941) appeared to indicate that the increased heat stability of the Vi antigen in these vaccines was to some extent due to dehydration. This suggested the possibility that by reason of interference with the normal water content of the bacterial cell, Vi antigen might prove to be more heat-stable in vaccines prepared with hypertonic saline than those prepared with an isotonic solution.

A comparison was therefore made of the effect of exposure at 25 and 37° C. on the agglutination reactions to pure Vi and O sera of merthiolate-killed and preserved bacilli in hypertonic saline, with those suspended in physiological saline.

Preliminary experiments showed that 32 % by weight of sodium chloride dissolved in distilled water was the highest concentration that could be maintained in solution at 0–2° C. without danger of the salt crystallizing out. A hypertonic saline suspension was consequently prepared by washing off a 24 hr. growth from agar with 32 % saline solution and adding an equal volume of merthiolate solution which had been rendered hypertonic by the addition of 32 % by weight of sterile sodium chloride. When sterile this suspension was centrifuged until all the cells had deposited, the supernatant fluid was discarded and the deposit resuspended in 32 % saline solution, containing a 1/10,000 concentration of merthiolate. The physiological saline suspension was prepared from the same culture and in the same manner as that of the hypertonic suspension with the exception that isotonic saline and isotonic merthiolate was used.

Both suspensions were adjusted to an opacity equivalent to 90×10^9 organisms per c.c., so that when the hypertonic suspension was diluted with distilled water until isotonic, the final count would be equivalent to that

Table 3. *Showing the agglutinogenic properties of acetone-dried bacilli, compared with those of suspensions in physiological saline, preserved with merthiolate, following storage at various temperatures*

Vaccine	Condition as stored	Storage temp. °C.	Period of storage	Rabbit	Dose in millions	Titre of antibodies in sera		
						H	O	Vi
Ty 2 in physiological saline. Sterilized 1/2,000 merthiolate and preserved with merthiolate 1/10,000 + colloidal silver 0·008 %	As a dry powder after being washed with acetone and dried in vacuo	0–2	3 months	B/1	3500	20,000	20,000	1200
		37	6 weeks	F/2	,,	5,000	10,000	500
Ty 2 in physiological saline. Sterilized with merthiolate 1/2000 and preserved with merthiolate 1/10,000	As a dry powder after being washed in acetone and dried in vacuo	37	8 days	C/3	,,	10,000	10,000	800
		37	3 months	X/1	,,	2,500	40,000	1400
		25	12 months	N/6	,,	10,000	5,000	700
	As a suspension in physiological saline solution plus 1/10,000 merthiolate	37	8 days	C/2	,,	5,000	10,000	<20
		37	10 days	F/3	,,	10,000	20,000	20
		25	5 months	W/3	,,	20,000	10,000	<20

Table 4. *Showing the agglutination reactions of merthiolate-killed* Bact. typhosum *bacilli suspended in physiological saline, compared with those in* 32 % *saline, following storage for various periods at* 23–25 *and at* 37° C.

		Physiological saline suspensions										32 % saline suspensions				
Storage temp. °C. ...		23–25 In months						37 In days				23–25 In months	37 In days			
Period of storage ...		1	2	3	4	5	6	1	2	3	5	6	7	12	30	50
Pure O serum dilutions, titre for O 901 =1/10,000	1/20	−	−	−	+	+	+	+++	+	+	+	−	−	−	±	±
	1/40	−	−	−	++	+++	++	+++	++	++	++	±	−	−	±	±
	1/80	−	−	−	+++	+++	+++	+++	+++	+++	+++	±	−	−	±	+
	1/160	−	−	+	++	++	++	++	+++	+++	+++	+	−	−	+	++
	1/320	−	−	+	++	++	++	++	+++	+++	+++	+	−	−	±	++
	1/640	−	−	++	++	++	++	++	++	++	++	+	−	−	±	+
	1/1,280	−	−	++	++	++	++	+	+	++	++	±	−	−	−	±
	1/2,560	−	−	+	+	+	+	±	±	±	+	±	−	−	−	±
	1/5,000	−	−	±	±	±	±	−	−	±	±	−	−	−	−	−
	1/10,000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pure Vi serum dilutions, titre for Watson =1/1,280	1/100	+++	+++	++	++	+	±	+	+	+	±	+++	+++	+++	+++	++
	1/200	++	+++	++	+	±	±	±	±	±	−	++	++	++	++	+
	1/400	++	++	+	±	±	−	±	±	±	−	+	++	++	+	±
	1/800	+	++	±	±	−	−	−	−	−	−	±	+	+	±	±
	1/1,600	−	±	−	−	−	−	−	−	−	−	±	±	±	−	−

of the T.A.B.C. vaccine at present issued for use in the Royal Navy, i.e. 2500×10^6 per c.c.

Both these suspensions were stored at 37 and 25° C. and examined periodically, the hypertonic suspension being rendered isotonic by dilution with 1/10,000 merthiolate in distilled water before its agglutination reactions were tested.

It will be seen from Table 4 that the physiological saline suspension showed complete loss of O resistance and a failure to agglutinate with Vi antibody after being retained at 37° C. for 5 days, whereas the hypertonic suspension still showed a considerable degree of O resistance and only a slight reduction of agglutinability with Vi antibody after 50 days' exposure to this temperature.

VII. The immunogenic properties of 32 % saline suspensions after being stored at 37 and 25° C.

In view of the claims made by Felix (1941) for alcohol as a preservative of Vi antigen in typhoid vaccine, and in order to assess the value of 32 % saline solution in this respect, the immunogenic effect in the rabbit of three different types of typhoid vaccine were compared, after they had been stored at 37° C.

The three types consisted of either alcohol or merthiolate-killed bacilli suspended in (*a*) 1/10,000 merthiolate in physiological saline solution, (*b*) 1/10,000 merthiolate in 32 % saline solution, and (*c*) 25 % alcohol in physiological saline solution. In addition, some of the vaccines tested were prepared with alcohol-killed or merthiolate-killed baccilli which before being suspended in any of these three solutions were washed in acetone and dried in vacuo, for there were theoretical grounds for the belief that such treatment might enhance the preservative action of 32 % saline solution for Vi antigen. In all, seven different vaccines were prepared, and Table 5 shows the details of the preparation of each vaccine, the conditions under which it was stored, and the immunogenic response it invoked in the rabbit. Those vaccines which consisted of suspensions in 32 % saline solution, before being injected into the rabbit, were first rendered isotonic by dilution with distilled water containing 1/10,000 concentration of merthiolate, and any further dilution necessary to allow the required dose to be administered in a suitable volume was made with physiological saline plus 1/10,000 merthiolate. The remaining vaccines if they required dilution before being administered, were diluted with a solution of the same consistency as that with which they had been prepared. The results of this experiment are shown in Table 5, and it will be seen that the only vaccines that induced any significant Vi antibody response after being stored for 21 days were those prepared with 32 % saline solution. Five days' storage was sufficient to destroy almost completely the Vi antibody stimulating properties of a vaccine preserved with merthiolate 1/10,000 in physiological saline, and in view of this result the response induced by the vaccines prepared with 32 % saline after 50 days' storage is noteworthy. The response obtained with

alcohol-preserved vaccines was not any better than that obtained with vaccines preserved with merthiolate in physiological saline.

The protective value of some of these sera was later tested and compared by means of passive protection experiments in mice, and it was found (see Table 9) that the protective value was proportional to their Vi agglutinin titre, from which it can be concluded that these agglutinins were representative of true Vi antibody. It would appear, therefore, that if the potency to stimulate Vi antibody is considered as the only criterion of typhoid vaccine, then at 37° C., the life of a vaccine preserved with 1/10,000 merthiolate in 32 % saline is at least ten times longer than that of a vaccine preserved with 1/10,000 merthiolate in physiological saline.

Table 5. *Showing the agglutinogenic properties of various vaccines following storage at* 37° C.

Vaccine used for immunization						Titre of antibodies in sera		
Method of sterilization	Condition as stored	Opacity	No. of days at 37° C.	Rabbit	Dose in millions	H	O	Vi
5 % alcohol in physiological saline	Washed and resuspended in physiological saline +25 % alcohol	$2{\cdot}5 \times 10^9$ per c.c.	21	L/8	3500	160	40,000	<20
			30	W/2	„	80	40,000	20
	Washed in acetone, dried in vacuo and resuspended in 32 % saline +1/10,000 merthiolate	90×10^9 per c.c.	50	D/1	„	1,280	10,000	200
0 % alcohol in physiological saline	Washed in acetone, dried in vacuo and resuspended in 32 % saline +1/10,000 merthiolate	90×10^9 per c.c.	21	L/6	„	40,000	40,000	500
Merthiolate 1/2,000 in physiological saline	Washed in acetone, dried in vacuo, and resuspended in physiological saline +25 % alcohol	90×10^9 per c.c.	3	X/3	„	160	40,000	80
	Washed in acetone, dried and resuspended in physiological saline +1/10,000 merthiolate	90×10^9 per c.c.	3	X/2	„	5,000	40,000	100
	Washed in acetone, dried and resuspended in 32 % saline +1/10,000 merthiolate	90×10^9 per c.c.	50	W/1	„	10,000	20,000	160
	Washed and resuspended in physiological saline +1/10,000 merthiolate	90×10^9 per c.c.	5	N/2	„	20,000	80,000	20
			10	N/1	„	10,000	20,000	<20
Merthiolate 1/2,000 in 32 % saline	Washed in 32 % saline and resuspended in 32 % saline +1/10,000 merthiolate	90×10^9 per c.c.	21	N/3	„	20,000	10,000	1000
			50	W/5	„	20,000	10,000	160

Only sufficient time has elapsed to allow the immunization of rabbits W/7 and N/7 with a merthiolate-killed and preserved suspension in 32 % saline which had been stored at 23–25° C. for 6 and 7 months respectively. For reasons of comparison the reactions exhibited by these rabbits following immunization are shown in Table 2, and the protective quality of serum W/7 as titrated in mice in Table 9. The results obtained further confirm the preservative property of 32 % saline for Vi antigen.

The experiments made with alcohol-preserved suspensions of killed typhoid bacilli were so few that it would be unwise to draw any definite conclusions from their results with regard to its preservative property for Vi antigen at

temperatures of 23–25° C. They failed, however, to demonstrate that 25 % alcohol in physiological saline could prevent the rapid deterioration of Vi antigen at a temperature of 37° C.

VIII. The effect of sterilizing by heating at 60° C. on a 32 % saline suspension, compared with that of a suspension in physiological saline

As a further test of the heat stability of Vi antigen in bacilli, suspended in 32 % saline solution, an experiment was made to compare the reactions of such a suspension after it had been sterilized by heating at a temperature of 60° C., with those of a similarly treated suspension in physiological saline.

Two suspensions, each of an opacity equivalent to 90×10^9 organisms per c.c., were prepared from the same culture, one with 32 % saline and the other with physiological saline solution. Some samples of each suspension were heated for 2 hr. and others for 4 hr. in a water bath at 60° C. When cool sterility tests were made, and all samples tested, including those that had been heated for only 2 hr., were found to be sterile. The effect of this heat treatment on the Vi antigen of these suspensions was examined by the same methods as those employed in the earlier experiments, namely, a study of their agglutination reactions in the presence of O and Vi antibody, and their immunogenic effect in the rabbit.

The results of the agglutination tests both before and after being heated are shown in Table 6; the immunogenic response in the rabbit following immunization with samples heated for 4 hr. is in Table 7, and the protective value of the sera thus produced for mice against infection with $8 \times$ A.L.D. of a live suspension of Ty 2 is in Table 9. It will be seen that the results obtained with these two suspensions are in striking contrast and further confirm the property of 32 % saline of inducing a remarkable degree of heat stability in Vi antigen, an antigen hitherto considered to be extremely heat labile.

IX. The immunogenic properties of merthiolate-killed, acetone-dried typhoid bacilli when suspended in tetanus formol-toxoid

As a war measure men and women of the fighting forces are being immunized against tetanus. Ramon & Zoeller (1927) showed that when tetanus toxoid is administered together with typhoid vaccine, the response to the toxoid is greater than when it is given alone, and for this reason a combined T.A.B.C.-tetanus-formol-toxoid mixture is now being employed for the immunization of personnel in the Royal Navy.

The method used is that recently described by Maclean & Holt (1941). Two doses each of 1 c.c. of a vaccine-toxoid mixture are given subcutaneously with a 6 weeks' interval between each dose. In order that the usual first dose or the usual second dose of the vaccine should be contained in 1 c.c., two different mixtures are supplied. These are prepared by suspending heat-killed

Table 6. *Showing the effect of heating at* 60° C. *on the agglutination reactions of two different suspensions of* Ty 2

		Ty 2 in physiological saline			Ty 2 in 32 % saline			
		Unheated	2 hr. at 60° C.	4 hr. at 60° C.	Unheated	2 hr. at 60° C.	4 hr. at 60° C.	Ty O 901
Serum dilutions,	1/40	−	+ +	+ + +	−	+ +	+ +	+ + +
pure O serum	1/80	−	+ +	+ +	−	+	+ +	+ + +
titre for O 901	1/160	−	+ +	+ +	−	±	+	+ + +
=1/10,000	1/320	−	+ +	+ +	−	±	±	+ +
	1/640	−	+	+ +	−	±	±	+ +
	1/1,280	−	+	+ +	−	±	±	+ +
	1/2,560	−	±	+	−	−	−	+ +
	1/5,000	−	±	±	−	−	−	+
	1/10,000	−	−	−	−	−	−	±
Serum dilutions,	1/20	+ + +	+ +	+ +	+ + +	+ + +	+ + +	−
pure Vi serum	1/40	+ + +	+ +	+	+ + +	+ + +	+ + +	−
titre for Watson	1/80	+ + +	+	±	+ + +	+ + +	+ + +	−
=1/1280	1/160	+ + +	±	−	+ + +	+ + +	+ +	−
	1/320	+ +	−	−	+ +	+ +	+	−
	1/640	+	−	−	+	+	±	−
	1/1,280	±	−	−	±	±	±	−
	1/2,560	−	−	−	−	−	−	−

Table 7. *Showing the effect of sterilization by heating at* 60° C., *on the agglutinogenic properties of two different suspensions of* Ty 2

	Opacity	Rabbit	Dose in millions	Antibodies in each serum		
				Vi	O	H
Ty 2 in physiological saline sterilized by heating at 60° C. for 4 hr.	90×10^9 per c.c.	Z/1	3500	40	40,000	40,000
Ty 2 in 32 % saline sterilized by heating at 60° C. for 4 hr.	90×10^9 per c.c.	L/9	3500	1200	20,000	10,000

T.A.B.C. of ten times the normal strength in the toxoid in two different proportions as follows:

1st dose, mixture 1		2nd dose, mixture 2	
B. typhosus	500×10^6	*B. typhosus*	1000×10^6
B. para-typhosus A, B and C	250×10^6 of each	*B. para-typhosus* A, B and C	500×10^6 of each
Tetanus formol-toxoid	0·9 c.c.	Tetanus formol-toxoid	0·9 c.c.

While the beneficial effect of this procedure with regard to the production of an active immunity against tetanus cannot be doubted and has recently been confirmed by Maclean & Holt (1941), its effect on the Vi antibody-stimulating property of the vaccine was undetermined.

Tetanus formol-toxoid is prepared by growing *Cl. tetani* in broth until toxin has been formed. The broth is then filtered free from organisms, and 0·3 % formalin is added to the filtrate. The formalized filtrate is incubated at 37° C. until the toxoiding process is complete, and when complete any free formalin remaining is neutralized by adding approximately 0·3 % of sodium metabisulphite. Finally, the broth is filtered again and 0·5 % of phenol is added as a preservative. A consideration of this outline of the preparation of tetanus formol-toxoid indicates that the effect on the Vi antigen, if any, would be detrimental.

It will be seen that in addition to any effect the broth or the sodium metabisulphite might have, there are two ingredients in the formol-toxoid which are known to be harmful to Vi antigen, namely, formalin and phenol (Felix & Bhatnagar, 1935). While it is possible that neutralization by means of sodium metabisulphite might prevent any injurious action of the formalin, that of phenol would be almost certain to have an effect.

Unfortunately, it is not possible to replace phenol by merthiolate as a preservative of formol-toxoid, since the bacteriocidal and bacteriostatic properties of merthiolate are destroyed by derivatives of sulphur, and therefore it cannot be used in the presence of sodium metabisulphite. However, Bhatnagar, Speechly & Singh (1938), working with a phenol-killed-phenolized suspension of the V strain Vi 1, found that when freshly prepared the sensitivity of the suspension to Vi antibody was satisfactory, and it was only after a lapse of time that it became insensitive. It appeared therefore that the inhibiting action of phenol on the Vi antibody stimulating properties of Vi antigen might be avoided if merthiolate-killed acetone-dried bacilli were suspended in the formol-toxoid immediately before being administered, and injected before the phenol had had sufficient time to produce its effect. To test this hypothesis the following experiment was carried out.

Merthiolate-killed acetone-dried bacilli, which had been stored in the dry state for 7 months at room temperature, were suspended in tetanus formol-toxoid. It was found that if the formol-toxoid was added to the powder and the mixture allowed to stand for about 3 min., and then gently shaken, an even suspension was quickly and easily attained. This preparation having been made, the vaccine-toxoid mixture was allowed to stand at room tem-

perature. Two rabbits were injected, one with mixture that had stood for 10 min., and the other with the same mixture that had stood for 4 hr. This procedure was repeated at 4-day intervals until each rabbit had received three doses. The first dose consisted of 0·5 c.c. of toxoid containing 625×10^6 *Bact. typhosum*, the second 1·0 c.c. of toxoid and 1250×10^6 *Bact. typhosum*, and the third 1·0 c.c. of toxoid containing 2500×10^6 *Bact. typhosum*. It will be seen that the ratio of the total number of organisms to toxoid in the first two doses was approximately the same as that in 'mixture 1', and in the third dose the same as that in 'mixture 2'.

Since it was possible that the broth basis of the toxoid might itself exert an effect, a similar experiment was made in which the formol-toxoid was replaced with physiological saline solution to which 0·3 % of formalin, 0·5 % phenol and sufficient sodium metabisulphite to neutralize the formalin, had been added.

Table 8. *Showing the agglutinogenic properties of acetone-dried* Bact. typhosum *bacilli when suspended in tetanus formol-toxoid*

Vaccine used for immunization		Rabbit	Titre of antibodies in sera		
			H	O	Vi
Ty 2 sterilized with merthiolate 1/2000, in physiological saline, washed in acetone and dried in vacuo. Stored in the dried state for 7 months at room temperature	Injected 10 min. after being resuspended in tetanus formol-toxoid	X/5	10,000	40,000	1400
	Injected 4 hr. after being resuspended in tetanus formol-toxoid	L/4	20,000	10,000	80
	Injected 4 hr. after being resuspended in physiological saline +0·3 % formalin +0·4 % sodium metabisulphite +0·5 % phenol	L/5	5,000	10,000	40

The immunogenic reactions of these rabbits are shown in Table 8. It will be observed that the Vi agglutinin response of the rabbits immunized with the 4 hr. old mixtures was insignificant while that of X/5 immunized with 10 min. old mixture was extremely good. However, when the protective power of X/5 serum was titrated in mice against experimental infection with a live suspension of the strain Ty 2 (see Table 9), it was found that this serum with a Vi agglutinin titre of 1/1400 afforded no more protection than a serum L/6 which had a Vi agglutinin titre of only 1/500. The degree of protection afforded by serum X/5 was therefore approximately one-third of its expected value. This result shows that these Vi agglutinins were deficient in protective immune body and were not indicative of true Vi antibody. This phenomenon is very similar to that which occurs when rabbits are immunized with formolized suspensions of *Bact. typhosum* (Felix & Bhatnagar, 1935). It has been shown by Henderson (1939) that the action of formalin on Vi antigen is to modify its molecular structure in such a way as to lead to the production, when used for immunization, of an incomplete antibody molecule. It must therefore be concluded that exposure of Vi antigen to the effects of tetanus toxoid even

Table 9. *Showing the comparative protective value of variously produced anti-typhoid sera*

Suspension used for immunization	Storage temp. °C.	Period stored	Rabbit	Titre of antibodies in serum H	O	Vi	Dose of serum c.c.	Result using 8 × A.L.D. Exp. 1	Exp. 2	Exp. 3
Colloidal silver-killed bacilli, resuspended in physiological saline +0·004 % colloidal silver	25	5 months	3/B	5,000	10,000	<20	0·5	2/10	—	—
							0·25	2/10	—	—
	0–2	7 months	D/B	10,000	10,000	1280	0·5	9/10	—	—
							0·25	10/10	—	—
Merthiolate-killed bacilli, suspended in physiological saline and preserved with merthiolate 1/10,000 + 0·008 % colloidal silver	23–25	5 months	2/B	5,000	5,000	80	0·5	2/10	—	—
							0·25	2/10	—	—
	0–2	6 months	D/D	5,000	5,000	1280	0·5	10/10	—	—
							0·25	8/10	—	—
Merthiolate-killed bacilli suspended in physiological saline and preserved with merthiolate 1/10,000	0–2	1 year	H/2	20,000	10,000	1280	0·25	—	10/10	—
							0·10	—	7/10	—
	23–25	5 months	W/3	20,000	10,000	<20	0·25	—	0/10	—
	37	10 days	N/1	10,000	20,000	<20	0·25	—	1/10	—
	37	5 days	N/2	20,000	80,000	20	0·25	—	2/20	2/10
Merthiolate-killed bacilli suspended in 32 % saline + 1/10,000 merthiolate	37	21 days	N/3	20,000	10,000	1000	0·25	—	9/10	—
							0·10	—	9/10	—
	25	6 months	W/7	10,000	10,000	1400	0·25	—	—	10/10
							0·10	—	—	7/10
Merthiolate-killed acetone-dried bacilli stored in the dry state	25	1 year	N/6	10,000	5,000	700	0·25	—	—	10/10
Alcohol-killed bacilli, washed and resuspended in physiological saline + 25 % alc.	37	21 days	L/8	160	40,000	<20	0·25	—	0/10	—
Alcohol-killed acetone-dried bacilli suspended in 32 % saline + 1/10,000 merthiolate	37	21 days	L/6	40,000	40,000	500	0·25	—	10/10	—
							0·10	—	3/10	—
Physiological saline suspension of Ty 2 sterilized by heating at 60° C. for 4 hr.	—	—	Z/1	40,000	40,000	40	0·25	—	0/10	—
32 % saline suspension of Ty 2 sterilized by heating at 60° C. for 4 hr.	—	—	L/9	10,000	20,000	1200	0·25	—	9/10	—
							0·10	—	10/10	—
Merthiolate-killed acetone-dried bacilli suspended in tetanus formol-toxoid, and injected within 10 min. of being suspended	25	10 min.	X/5	10,000	40,000	1400	0·25	—	10/10	—
							0·10	—	4/10	—
Controls receiving 1/8 test dose suspension = Live suspension of Ty 2 in physiological saline								1/10	4/10	1/10
Controls receiving 1/5 test dose suspension								—	1/10	—
Controls receiving 1/4 test dose suspension								0/10	—	0/10
Controls receiving 1/3 test dose suspension								—	0/10	—
Controls receiving 1/2 test dose suspension								0/10	—	0/10

Exp. 1. Test dose suspension = 400×10^6. $1 \times$ A.L.D. = 50×10^6 = that dose which will kill at least 50 % of mice in 48 hr.
Exps. 2 and 3. Test dose suspension = 600×10^6. $1 \times$ A.L.D. = 75×10^6.
Denominator: Number of mice tested. Numerator: Number of mice surviving after 7 days.

for 10 min. is sufficient to affect adversely its immunogenic properties. It is probable that this rapid effect is brought about by the presence of formalin or the compounds which result from its neutralization with sodium metabisulphite.

These experiments suggest that the detrimental action of phenol on Vi antigen can be minimized by reducing the time of exposure of the antigen to this antiseptic.

X. DISCUSSION

These experiments show that *Bact. typhosum* suspended in physiological saline solution can be killed without any destruction of the Vi or O antigen by merthiolate or colloidal silver. As has been remarked previously the same result was obtained by Felix with alcohol and by Henderson with acetone. Suspensions in physiological saline of bacilli killed with merthiolate or colloidal silver and preserved with one or other of these chemicals when stored at 0–2° C. retained their Vi antibody-stimulating property for the rabbit unimpaired for 1 year, but at 23–25° C. this property was lost after 4–5 months.

The pronounced effect of temperature on the rate of loss of Vi antigen from a vaccine has been demonstrated further by experiments in which it was shown that an exposure of these vaccines for 4 days to a temperature of 37° C., or for 4 hr. at 60° C., produced approximately the same effect as 4 months at 23–25° C.

It therefore appeared that if the rapid deterioration of Vi antigen was to be prevented at temperatures of 23–25° C., some method would have to be found by which its heat stability could be increased. It has been shown that there are two methods available by which this can be accomplished, namely, desiccation of *Bact. typhosum* bacilli following treatment with acetone, or by suspending the bacilli in 32 % saline.

That the degree of heat stability conferred on Vi antigen by the former of these two methods is of a high order has been shown by experiment in which it was found that acetone-washed and dried bacilli showed no loss of Vi potency for the rabbit either following 3 months' storage at 37° C., or 1 year at 23–25° C.

It is probable therefore that such dried vaccines would retain their immunogenic properties for both Vi and O antibody almost indefinitely, even when stored at temperatures of 23–25° C. The production of vaccine by this process would be very expensive, however, and for technical reasons difficult to operate on a large scale. On the other hand, the alternative method, namely, that of suspending the organisms in 32 % saline, is an extremely simple and inexpensive process which offers no difficulties to large-scale production.

Although the experiments indicate that 32 % saline is not so effective for preserving Vi antigen as that of complete desiccation, they show that killed bacilli suspended in this solution will; when exposed to a temperature of 37° C., withstand an exposure of 21 days without any apparent loss of the property to stimulate Vi antibody formation in the rabbit, and even after

50 days' exposure they are very much superior in this respect to suspensions in physiological saline which have been exposed to the same temperature for only 5 days. The degree of heat stability afforded to Vi antigen at 37° C. by 32 % saline, is therefore at least ten times as great as that pertaining in a suspension in physiological saline. If the same ratio held true at 23–25° C., suspensions in 32 % saline should retain their Vi antibody immunogenic property for at least 20 months. Sufficient time has not yet elapsed to allow it to be proved that this is so, but the result obtained in rabbits immunized with a vaccine which had been prepared with merthiolate and 32 % saline and stored at 23–25° C. for 6 months, and 7 months, lends support to such a conclusion.

The experiments which have been described were made with the object, inter alia, of discovering a method by which an improved antityphoid vaccine could be prepared, capable of stimulating Vi and O antibody formation in man even after it had been stored for 12–18 months at 23–25° C. If either of the two methods of preserving Vi antigen evolved as the result of this work are adhered to in principle, it is believed that a vaccine to meet this specification can be produced. A description of the technique for the preparation, supply, and administration of two new types of T.A.B.C. vaccine will be found at the end of this paper.

The results of the experiments carried out with tetanus formol-toxoid raise the question as to whether it is advisable to continue the present method of combined immunization against tetanus and typhoid. There is good reason to believe that even heat-killed-phenolized vaccine when given alone is capable of inducing some Vi antibody response in man. Felix *et al.* (1941) have shown that 6–7 % of men, following immunization with this type of vaccine, developed Vi agglutinins in their sera, and Bhatnagar (1938) found that 72·2 % of patients suffering from typhoid fever who previously had been inoculated with heat-killed-phenolized vaccine, developed a Vi agglutinin titre of 1/100 or higher, whereas only 7·7 % of an uninoculated group showed an equivalent rise of titre. It would seem therefore that in the majority of subjects, following immunization with heat-killed-phenolized vaccine, the tissues are sensitized to Vi antigen, although circulating Vi antibody cannot be detected in the blood by the usual in vitro tests. Further, Felix (1941) states that the Vi antigens of *Bact. paratyphosum* A and B are less heat- and phenol-labile than Vi antigen. It is therefore reasonable to assume that heat-killed-phenolized vaccine will produce a relatively better immunity against infections of *Bact. paratyphosum* A and B than against *Bact. typhosum* or *Bact. paratyphosum* C.

Although, experimentally, heat-killed-phenolized T.A.B.C. vaccine compared with the newer types of vaccine is a poor agent for immunization against typhoid infections, its beneficial value in the field has been shown by the marked lowering of the incidence of typhoid and paratyphoid infections in both the Royal Navy and British Army ever since it has been employed prophylactically by these forces.

We have, however, no extensive experience of its value in the field when mixed with tetanus formol-toxoid, since this method of immunization has only been practised for a comparatively short time.

It has been shown that the prophylactic value of this vaccine may be due to its ability to sensitize the tissues to Vi antigen, and that formalin or its compounds present in tetanus formol-toxoid have a detrimental action on the immunogenic properties of this antigen. It is therefore possible that the ability of heat-killed-phenolized vaccine to sensitize the tissues to Vi antigen when given alone may be lost when it is mixed with tetanus formol-toxoid.

Henderson (1939*a*), however, has shown that when the virulence of *Bact. typhosum* for mice is increased approximately a thousandfold by the addition of mucin, O antibody alone is capable of protecting these animals against a moderate number of fatal doses of a fully virulent Vi plus O strain. The efficacy in prophylaxis of heat-killed-phenolized vaccine therefore may depend entirely on its O antigen content, and the subsequent development in man of its homologous antibody following immunization with this vaccine.

Further experimental work is now being undertaken with the object of ascertaining why this type of vaccine, which experimentally produces no Vi antibody response in the rabbit, can apparently induce a significant degree of active immunity against infections with V strains of *Bact. typhosum*, both in the mouse (Schütze, 1936) and in man.

XI. SUGGESTED TECHNIQUE FOR THE PREPARATION, SUPPLY, AND ADMINISTRATION OF TWO NEW TYPES OF T.A.B.C. VACCINE

Selection of strains

The presence of specific Vi antigens of their own in certain strains of *Bact. paratyphosum* A and B was first described by Felix & Pitt (1936), while Kauffmann (1935) has shown that there are strains of *Bact. paratyphosum* C which possess the same Vi antigen as that present in V strains of *Bact. typhosum*. Felix (1941) states that the Vi antigens of *Bact. paratyphosum* A and B and their homologous antibodies in all probability bear the same relationship to these organisms as do Vi antigen and its antibody to *Bact. typhosum*. It is therefore essential if full benefit is to be obtained from these new vaccines, that they should be prepared from selected strains known to contain these antigens. Felix (1941) has given some detailed advice as to the strains that should be employed.

(1) *Dried T.A.B.C. vaccine*

Preparation. Cultures grown on beef bouillon agar *p*H 7·4, incubated for 24 hr. at 37° C., are washed off and suspended in physiological saline solution. An equal volume of *Merthiolate Solution* no. 45 is added to this suspension, mixed, and the mixture placed in a refrigerator. Sterility tests are made daily by the technique already described. When sterile, a sample of the suspension is removed and a bacterial count made. The suspension is then centrifuged

until all the cells have deposited, the supernatant fluid is discarded, and the cells resuspended in physiological saline plus 1/10,000 merthiolate, i.e. physiological saline 9 parts, *Merthiolate Solution* no. 45 1 part, sufficient of this solution being added to allow of a final opacity equivalent to 50×10^9 organisms per c.c. At this stage the suspension is again tested for sterility. The four different suspensions, all of which are prepared in the same manner, are now mixed together in the proportion of 2 parts of *Bact. typhosum* suspension to 1 part each of *Bact. paratyphosum* A, B and C suspension, and 1 c.c. of the mixture is measured into a 1 oz. screw-cap bottle (Mackie & Macartney, 1938*a*), and 1 c.c. of acetone is added. The addition of the acetone will cause the cells to agglutinate and sediment rapidly. This mixture is centrifuged, and when the cells have deposited the supernatant fluid is removed and the sediment washed twice with pure acetone. Following the last-washing, the bottle now containing only the acetone-washed and deposited cells is connected by means of a sterile metal screw-cap filter-top (Mackie & Macartney, 1938) (see Fig. 1) to a high vacuum pump, a large container filled with calcium chloride being interposed between the 1 oz. bottle and the pump. The bottle is now completely evacuated, and the cells are thereby dried within a few minutes. By the use of Carrel tubes large numbers of bottles can be evacuated in one operation (see Fig. 2). When the vaccine has been completely dried each bottle is disconnected and capped with a sterile metal cap fitted with a thick rubber liner. The top of the cap is perforated so as to expose a circular area of 7 mm. in diameter of the inner rubber liner. The bottle is finally completely sealed by covering the cap and neck of the bottle with a *vis-cap*.

Supply. Each bottle will contain sufficient dried vaccine when resuspended in physiological saline to make 20·0 c.c. of T.A.B.C. vaccine containing: *Bact. typhosum*, 1000×10^6 per c.c.; *Bact. paratyphosum* A, B and C, 500×10^6 of each per c.c. Therefore, with each bottle of vaccine it will be necessary to supply a 1/10,000 solution of merthiolate in sterile physiological saline. This should be issued in rubber-capped vaccine bottles in 25·0 c.c. amounts. To conform with the Therapeutic Substances Act, samples from each batch of vaccine, and from each batch of saline and merthiolate solution, would require to be tested for sterility and toxicity before issue.

Administration. Immediately before administration the vis-cap is removed from the bottle containing the dried vaccine. The top of the metal cap and the small exposed area of the inner rubber liner are swabbed with an antiseptic, and a sterile hypodermic needle plunged through the liner. Using a 20 c.c. syringe, with the usual aseptic precautions, 20 c.c. of merthiolate and physiological saline solution is removed from its bottle and injected into the bottle containing the dried vaccine, the air in this bottle being allowed to escape by means of the first hypodermic needle which is left in situ during this operation. Both needles are now removed, and the bottle allowed to stand for 15 min. Finally, it is shaken until all the powder has gone into suspension. When completely suspended the vaccine is ready for use. It is recommended that

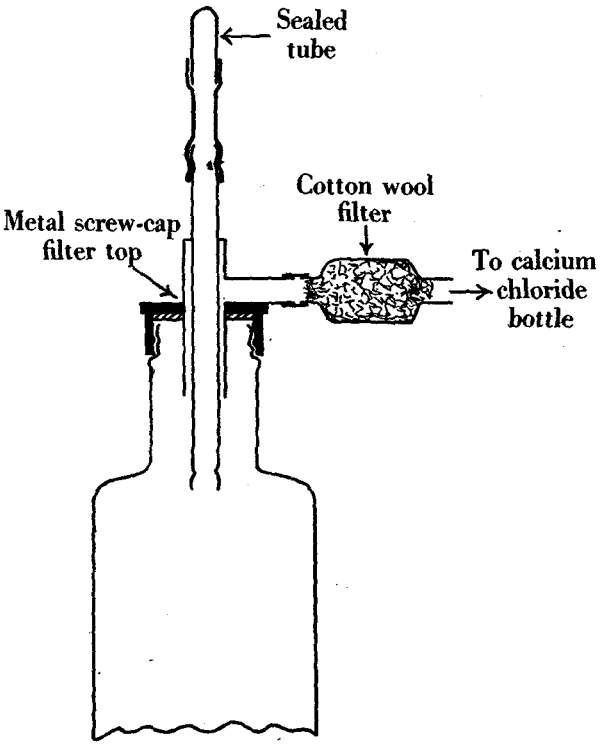

Fig. 1

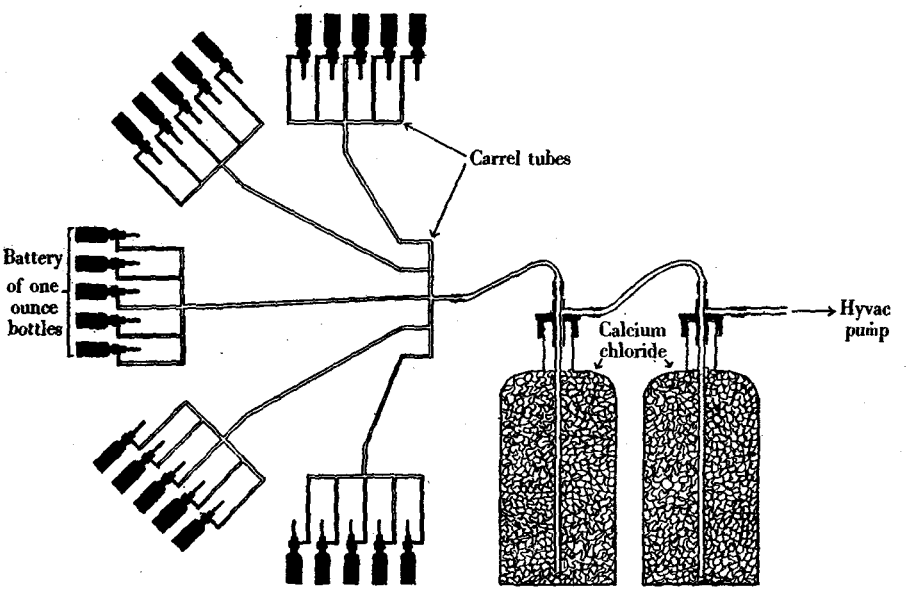

Fig. 2

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the usual two doses of 0·5 and 1·0 c.c. should be given with an interval of 7–10 days between each dose.

(2) *T.A.B.C. vaccine using* 32 % *saline solution*

Preparation. Two solutions are required.

Solution 1 consists of *Merthiolate Solution* no. 45 in which 320 g. of sodium chloride is dissolved and made to 1 l. In preparing this solution it is advisable first to measure the weight of salt required into a sterile screw-capped bottle or flask, and sterilize this in the autoclave. When cool add the merthiolate solution and shake to dissolve.

Solution 2 is prepared by adding 1 part of solution 1 to 9 parts of sterile 32 % saline solution.

The 24 hr. growth from an agar culture is washed off and suspended in 32 % saline, and to this is added an equal volume of solution 1. This suspension is placed in a refrigerator and sterility tests are made daily. When sterile a sample is removed and a bacterial count made. The suspension is then centrifuged to deposit all the cells, the supernatant fluid is discarded, and the deposit resuspended in sufficient of solution 2 to allow of an opacity equivalent to 90×10^9 organisms per c.c. On completion of this procedure, further sterility tests are made. The four suspensions, all of which are prepared in the same manner, are now mixed together in the proportion of 2 parts of typhoid suspension to 1 part each of the others. The mixture is finally filled into 1 c.c. sterile glass ampoules.

Supply. Each ampoule is supplied together with a rubber-capped vaccine bottle containing 25 c.c. of 1/10,000 merthiolate in sterile distilled water. Before being issued, samples from each batch of vaccine, and from each batch of distilled water and merthiolate solution, are tested for sterility and toxicity.

Administration. Using a sterile 1·0 c.c. syringe and with the usual precautions, 0·7 c.c. of the vaccine is removed from the ampoule and injected into the bottle containing the distilled water and merthiolate solution. The bottle is now well shaken, and the vaccine is ready for use. The same dosage is recommended as for that of the other vaccine.

It will be seen that the preparation of the dried vaccine involves the use of a considerable quantity of specialized apparatus, and that its administration would require extreme care if systemic reactions are to be avoided. There is, however, the possibility that this type of vaccine might be found of value in tropical and subtropical climates under circumstances where even cool storage is unobtainable. In view of the easier and cheaper method of preparing vaccine with 32 % saline, and its simpler and safer method of administration, it is recommended that the dried vaccine should be reserved for use only in those special circumstances which have been mentioned.

XII. SUMMARY

1. V strains of *Bact. typhosum* can be sterilized by means of merthiolate or colloidal silver without destructive effect on either the Vi or O antigens.

2. Suspensions in physiological saline sterilized and preserved by the addition of either merthiolate or silver retain their property of stimulating Vi antibody formation in the rabbit for at least one year if stored at 0–2° C., but when stored at 23–25° C. this property is lost after 4 months' storage.

3. The Vi antibody-stimulating property of a merthiolate-killed and preserved suspension in physiological saline is almost completely destroyed by an exposure of 5 days at 37° C. or 4 hr. at 60° C.

4. *Bact. typhosum* bacilli killed with merthiolate or alcohol, and dried with acetone, show no loss of Vi antigen when stored in the dry state for 1 year at 23–25° C., or after 3 months at 37° C.

5. Suspensions sterilized and preserved with merthiolate in 32 % saline will withstand 50 days' storage at 37° C., or 7 months at 23–25° C., without significant loss of their Vi antibody-stimulating properties.

6. Suspensions in 32 % saline can be sterilized by an exposure to 60° C. for 2 hr., and even after 4 hr. exposure at this temperature, show no loss of Vi antigen content.

7. Suspensions killed by merthiolate or alcohol, and preserved by the addition of 25 % alcohol in physiological saline, failed to stimulate Vi antibody formation in the rabbit after being stored for 20 days at 37° C.

8. The application of these findings to the production of a stable T.A.B.C. vaccine of enhanced immunological qualities is discussed.

9. The effect of mixing *Bact. typhosum* with tetanus formol-toxoid has been examined, and the advisability of employing heat-killed-phenolized vaccine mixed with tetanus formol-toxoid for combined immunization against tetanus and typhoid infections is discussed.

10. A suggested technique for the preparation, supply, and administration of two new types of T.A.B.C. vaccine is described.

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