THE REPRODUCIBILITY OF EXTINCTION TIME ESTIMATES

PART III. STUDIES OF SUSPENSIONS OF TEST ORGANISMS

BY A. M. COOK, K. J. STEEL AND B. A. WILLS

From the Department of Pharmaceutics, School of Pharmacy, University of London

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VARIATIONS in the resistance of suspensions or cultures of the test organism used in the evaluation of bactericides have been reported by several workers[1-3]. Cook and Steel[4] indicated that this variability might be reduced by the use of a single suspension, stored between experiments at room temperature.

The change in phenol resistance of suspensions during storage was investigated.

EXPERIMENTAL

Preliminary Experiments

The organisms used were, *Bacterium coli* (*Escherichia coli*), *Bacillus anthracis*, *Bordetella bronchisepticus*, *Mycobacterium smegmatis*, *Pseudomonas pyocyanea*, *Salmonella typhi*, *Shigella dysenteriae Type I*, *Staphylococcus aureus* and *Streptococcus fæcalis*. The strains used were those used by Cook[5].

Method

The organisms were grown in Roux bottles on Lemco agar for 48 hours. Suspensions were made by washing the organisms off the agar with 50 ml. of sterile distilled water and were stored in 100 ml. bottles. No attempt was made to wash the suspensions free from agar or nutrients.

Counts were made of the suspensions using the Miles and Misra[6] technique with tenfold dilutions and five replicate plates for each organism.

Table I shows the results of the initial counts and the counts after storage for a month at ambient room temperature.

TABLE I

VIABLE COUNT OF SUSPENSIONS STORED AT ROOM TEMPERATURE FOR 1 MONTH

Organism	Original count	Count after storage
Bacterium coli	$7·9 \times 10^{9}$	$5·4 \times 10^{8}$
Bacillus anthracis	$7·4 \times 10^{5}$	$6·0 \times 10^{4}$
Bordetella bronchisepticus ..	$2·4 \times 10^{10}$	$1·0 \times 10^{8}$
Mycobacterium smegmatis ..	$3·0 \times 10^{6}$	$1·1 \times 10^{7}$
Pseudomonas pyocyanea ..	$7·6 \times 10^{8}$	$5·2 \times 10^{7}$
Salmonella typhi	$8·0 \times 10^{9}$	$1·8 \times 10^{7}$
Streptococcus fæcalis	$3·6 \times 10^{8}$	$9·0 \times 10^{7}$

Other experiments had indicated that a constant temperature of 10° C. might result in an increase of the numbers of organisms surviving. Fresh suspensions were made and stored at $10° \pm 1°$ C. Counts were made at intervals on these suspensions.

The resistance of the organisms to several bacteriostatics were determined initially and after three months storage. This was done by inoculating agar plates containing various strengths of the bacteriostatics with drops of the suspensions, as described by Cook[5].

Figure 1 shows the reductions in the viable counts over the period of storage, and Table II shows changes in bacteriostatic resistance.

TABLE II

CHANGE IN BACTERIOSTATIC RESISTANCE OF VARIOUS ORGANISMS AFTER 3 MONTHS STORAGE

		Bact. coli	*B. anthracis*	*Bordetella bronchiseptica*	*Myco. smegmatis*	*Ps. pyocyanea*	*Salm. typhi*	*Shig. dysenteriæ*	*Staph. aureus*	*Strept. fæcalis*
Phenol	A	0·2	0·2	0·1	0·1	0·2	0·2	0·2	0·1	<0·1
	B	0·2	0·1	0·1	0·1	0·2	0·1	0·1	0·1	<0·1
Chloro-cresol	A	0·04	0·04	>0·02	>0·02	<0·08	0·04	0·04	0·04	0·08
	B	0·04	>0·02	>0·02	>0·02	0·08	0·04	>0·02	0·04	0·04
Phenyl mer-curic acetate	A	0·002	>0·0001	0·002	>0·0001	0·002	0·002	0·002	>0·0001	0·002
	B	0·0005	>0·0001	0·0005	>0·0001	0·002	0·0005	>0·0001	>0·0001	>0·0001
Cetrimide	A	>0·005	>0·005	>0·005	>0·005	>0·1	0·025	>0·005	>0·005	0·025
	B	>0·005	>0·005	>0·005	>0·005	0·1	>0·005	>0·005	>0·005	>0·005
Crystal violet	A	0·002	0·0005	<0·002	0·002	<0·002	<0·002	<0·002	0·0005	0·0005
	B	0·002	>0·0001	<0·002	0·0005	<0·002	<0·002	<0·002	0·0001	0·0005
Aminacrine hydrochloride	A	<0·08	<0·08	0·016	>0·002	0·08	0·016	>0·002	>0·002	>0·002
	B	<0·08	<0·08	>0·002	>0·002	0·08	>0·002	>0·002	>0·002	>0·002
Chloramine T	A	0·5	0·2	0·5	0·5	0·5	0·5	0·2	0·5	0·5
	B	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5

A = inhibiting concentration (expressed as percentage) initially.
B = ,, ,, (,, ,, ,,) after storage.

Investigations of unwashed suspensions of Bacterium coli *stored at different temperatures*

The strain of *Bacterium coli* used by Cook and Steel[4] had better survival figures on storage than any of the organisms used in the preliminary experiments and so it was decided to use this organism for further investigations.

The suspension was prepared by washing the 24 hour growth from several Roux bottles containing a peptone agar (1 per cent. peptone, 0·5 per cent. sodium chloride, 2 per cent. agar, pH 7·2). After shaking vigorously with glass beads, the suspension was centrifuged at 3000 r.p.m. for 1 minute, the supernatant removed and diluted to give an optical density equivalent to a total count of 2×10^9 per ml. Five ml. portions of the suspension were distributed in 5 ml. ampoules, which were sealed and divided into 5 batches for storage at temperatures of 4°, 10°, room temperature (c. 18° C.), 20° and 37° C. At intervals during the next six months, ampoules were opened and viable counts and extinction times determined by the methods outlined below.

The counting method used was that described by Miles and Misra[6]. A suitable number of drops of suspension was added to suitable volumes of sterile water. After thorough mixing, samples at two or more dilutions were dropped on to the surfaces of over-dried agar plates, the agar having the same composition as that used in preparation of the suspension. Dropping pipettes were of the same type and similar accuracy to those described by Cook and Yousef[7] and Cook[5]. Between 20 and 60 samples were taken at each dilution and the colonies were counted after incubation at 37° C. for between 12 and 15 hours.

Extinction times were determined by the method described by Berry and Bean[8] subject to the modifications put forward by Cook and Wills[3,9]. The bactericide solutions used contained 1·1 per cent. phenol in aqueous solution at 20° C. Each experiment consisted of between 10 and 20 replicate determinations—usually 15—and the results were expressed as mean single survivor times according to the analysis of Mather[10].

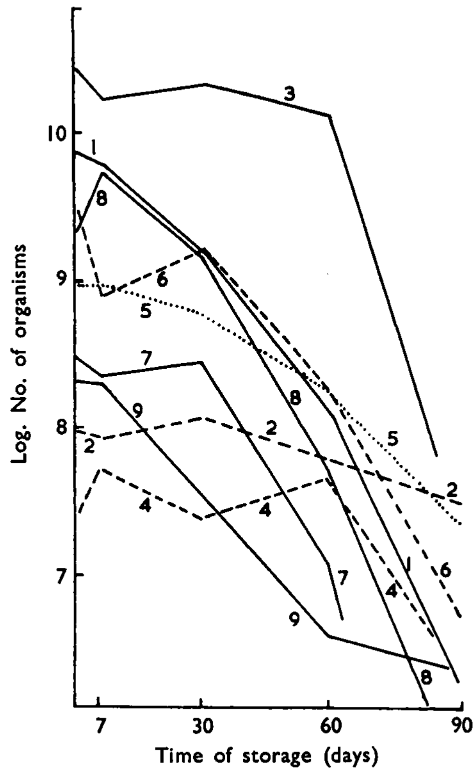

FIG. 1. Numbers of viable organisms per ml. in suspensions of different bacteria stored at 10° C. for various times. 1. *Bacterium coli*; 2. *Bacillus anthracis*; 3. *Bordetella bronchisepticus*; 4. *Mycobacterium smegmatis*; 5. *Pseudomonas pyocyanea*; 6. *Salmonella typhi*; 7. *Shigella dysenteriæ* (type I); 8. *Staphylococcus aureus*; 9. *Streptococcus fæcalis*.

The results of viable counts and extinction times of the suspension after varying periods of storage are shown in Table III. After the 23rd day of storage, the extinction times of the suspensions kept at 37° C. decreased so much that a lower phenol concentration of 0·7 per cent. had to be used in later experiments. The right hand column of the Table records extinction times to 1·1 per cent. phenol of a second laboratory strain of *Bact. coli* which was of lower phenol resistance and which was stored at room temperature in a stoppered bottle.

Investigation of washed suspensions of Bacterium coli *stored at different temperatures and in different containers*

The suspensions were prepared in the same way as before, but in

TABLE III

VIABILITY AND EXTINCTION TIMES TO 1·1 PER CENT. AQUEOUS PHENOL SOLUTION OF UNWASHED SUSPENSIONS OF *Bact. coli* (N.C.T.C. 5933) STORED AT DIFFERENT TEMPERATURES

Storage time (days)	4° C.		10° C.		Room temperature (*c.* 18° C.)		20° C.		37° C.		Other strain (18° C.)
	Viable count/ml.	Extinction time (min.)	Viable count/ml.	Extinction time (min.)	Viable count/ml.	Extinction time (min.)	Viable count/ml.	Extinction time (min.)	Viable count/ml.	Extinction time (min.)	
0	—	—	$1·94 \times 10^9$	—	—	—	—	—	—	—	13·2
1	—	—	—	—	—	—	—	—	—	—	16·2
2	—	—	—	—	—	32·8	—	—	—	—	—
3	$1·77 \times 10^9$	—	—	—	—	—	—	—	—	—	15·7
6	—	—	$1·89 \times 10^9$	—	$1·85 \times 10^9$	—	$1·81 \times 10^9$	—	$1·44 \times 10^8$	—	—
7	$1·86 \times 10^9$	28·2	—	—	—	—	—	—	—	19·6	10·3
9	—	—	—	23·4	—	—	$1·95 \times 10^9$	45·4	—	—	—
13	—	—	—	—	—	—	$1·68 \times 10^9$	31·4	—	—	—
17	—	—	—	—	—	31·5	—	—	—	—	9·05
20	—	21·0	—	—	—	—	—	—	—	2·67	—
23	—	—	—	—	—	—	—	—	—	1·55	—
28	—	—	—	28·3	—	—	—	—	—	—	—
37	—	33·4	—	—	—	—	—	32·0	$1·04 \times 10^7$	34·8	—
42	—	—	—	30·4	$1·62 \times 10^8$	21·2	—	—	—	19·9	—
57	—	—	—	—	—	23·6	—	14·7	$7·08 \times 10^5$	10·8	—
69	—	—	—	26·5	—	—	—	—	—	6·63	—
72	—	20·2	—	—	—	26·0	—	—	—	—	—
78	$9·20 \times 10^8$	—	$1·53 \times 10^8$	22·1	$5·90 \times 10^7$	—	$4·62 \times 10^8$	—	$2·76 \times 10^5$	—	—
90	$9·20 \times 10^8$	—	$1·73 \times 10^8$	—	$3·99 \times 10^7$	—	$1·74 \times 10^7$	—	$2·88 \times 10^5$	—	11·0
115	—	—	—	—	—	17·4	—	—	$1·88 \times 10^5$	—	—
118	—	17·6	—	21·4	—	—	—	—	—	—	—
125	—	—	—	—	—	—	—	8·00	—	—	—
136	—	—	—	—	—	—	—	—	$1·05 \times 10^5$	4·90	—
139	$5·55 \times 10^8$	—	$9·35 \times 10^7$	—	$1·25 \times 10^7$	—	$6·79 \times 10^6$	—	—	—	—
155	$8·34 \times 10^8$	—	$8·96 \times 10^7$	—	$1·03 \times 10^7$	—	$6·04 \times 10^6$	—	—	—	—
170	$2·70 \times 10^8$	13·6	$6·74 \times 10^7$	16·4	—	9·90	—	4·85	—	—	—
175	—	—	—	—	—	—	—	—	$1·35 \times 10^5$	5·63	—

addition the cells were finally washed by centrifuging and resuspending in water, repeating the process 4 times. The final suspension was stored at 4°, 10°, 20° and 37° C., in 100 ml. glass-stoppered bottles, approximately half filled, in 5 ml. ampoules containing 5 ml., and in 5 ml. ampoules which were filled with carbon dioxide after distribution of the suspension. In addition, a few ampoules were filled as nearly as possible to capacity with the suspension and a few were filled with only 1 ml. of suspension.

TABLE IV

VIABILITY AND EXTINCTION TIMES TO 1·1 PER CENT. AQUEOUS PHENOL SOLUTIONS OF WASHED SUSPENSIONS OF *Bact. coli* (N.C.T.C. 5933) STORED AT DIFFERENT TEMPERATURES

Storage time (days)	4° C.		10° C.		20° C.		37° C.
	Viable count/ml.	Extinction time (min.)	Viable count/ml.	Extinction time (min.)	Viable count/ml.	Extinction time (min.)	Viable count/ml.
0	—	—	$1·69 \times 10^9$	—	—	—	—
5	—	—	$1·65 \times 10^9$	—	$1·42 \times 10^9$	—	$1·12 \times 10^9$
9	—	—	—	—	—	—	$5·16 \times 10^8$
13	—	—	$1·56 \times 10^9$	—	$1·03 \times 10^9$	—	—
15	—	—	—	—	—	—	$2·06 \times 10^8$
22	—	—	$1·48 \times 10^9$	—	$9·66 \times 10^8$	—	$7·32 \times 10^7$
26	—	—	—	16·2	—	—	—
28	—	—	—	—	—	11·3	—
35	—	—	$1·27 \times 10^9$	—	$7·80 \times 10^8$	—	$1·24 \times 10^7$
50	$7·80 \times 10^8$	7·90	—	—	—	—	—
52	—	—	—	10·5	—	—	—
55	—	—	—	—	—	9·59	—
57	—	—	$7·32 \times 10^8$	—	$4·50 \times 10^8$	—	$4·40 \times 10^6$

The values of extinction times and viable counts listed in Table IV refer only to suspensions stored in bottles. At the end of 2 months a comparison of counts at different temperatures and in different containers was made. The results are recorded in Table V.

Investigation of washed suspensions of Staphylococcus aureus *stored at different temperatures and in different containers*

The organism used in these tests was *Staphylococcus aureus* (Oxford) N.C.T.C. No. 6571. The suspension was prepared as described above, the organisms being harvested from a medium containing 1 per cent. Lab Lemco, 1 per cent. peptone, 0·5 per cent. sodium chloride and solidified with agar (pH 7·2). Samples of the suspension were stored at 10°, room temperature, 20° and 37° C., and in the same range of containers as described above. In addition, 250 ml. plugged conical flasks containing about 50 ml. of suspension were stored at 10° and 37° C., a mark being filed on each flask at the liquid level to allow frequent adjustment to replace water lost by evaporation. Media used both in extinction time determinations and in the viable counts contained 1 per cent. Lab Lemco in addition to peptone. The results in Table VII were obtained from suspensions stored in bottles. Results of viable counts in all types of container at 3 different temperatures after storage for 2 months are shown in Table VI.

The time taken for visible colonies to appear increased with increased duration of storage, so that an incubation period of at least 48 hours

TABLE V

COMPARISON OF VIABLE COUNTS PER ML. OF WASHED *Bact. coli* SUSPENSIONS STORED UNDER DIFFERENT CONDITIONS FOR 57 DAYS

Storage temperature	10° C.			20° C.			37° C.				
Container	Bottle	Ampoule	Ampoule under CO_2	Bottle	Ampoule	Ampoule under CO_2	Bottle	Full ampoule	3/4 full ampoule	1/5 full ampoule	Ampoule under CO_2
Initial count ..	$7{\cdot}32 \times 10^8$	$7{\cdot}68 \times 10^8$	$1{\cdot}47 \times 10^7$	$4{\cdot}50 \times 10^8$	$2{\cdot}88 \times 10^8$	$5{\cdot}76 \times 10^6$	$4{\cdot}40 \times 10^6$	$6{\cdot}72 \times 10^5$	$2{\cdot}83 \times 10^6$	$3{\cdot}73 \times 10^6$	$2{\cdot}10 \times 10^2$
Count* after 48 hrs.	—	—	$1{\cdot}27 \times 10^7$	—	—	$2{\cdot}58 \times 10^7$	—	$1{\cdot}52 \times 10^7$	$8{\cdot}40 \times 10^6$	$6{\cdot}54 \times 10^6$	$3{\cdot}78 \times 10^7$

* Suspensions aerated, transferred to test tube and incubated for 48 hours at the temperature at which the ampoules were originally stored.

TABLE VI

COMPARISON OF VIABLE COUNTS OF WASHED *Staph. aureus* SUSPENSIONS, STORED UNDER DIFFERENT CONDITIONS FOR 56 DAYS

Storage temperature	10° C.				20° C.			37° C.					
Container	Bottle	Plugged flask	Ampoule	Ampoule under CO_2	Bottle	Ampoule	Ampoule under CO_2	Bottle	Plugged flask	Full ampoule	3/4 full ampoule	1/5 full ampoule	Ampoule under CO_2
Initial count ..	$1{\cdot}32 \times 10^6$	$8{\cdot}90 \times 10^6$	$2{\cdot}64 \times 10^6$	$1{\cdot}41 \times 10^4$	$2{\cdot}08 \times 10^8$	$1{\cdot}49 \times 10^6$	$6{\cdot}96 \times 10^3$	$1{\cdot}34 \times 10^6$	$5{\cdot}70 \times 10^6$	$1{\cdot}39 \times 10^6$	$5{\cdot}82 \times 10^5$	$5{\cdot}70 \times 10^4$	0
Count* after 48 hrs.	—	—	—	$1{\cdot}79 \times 10^4$	—	—	$3{\cdot}6 \times 10^2$	—	—	$1{\cdot}73 \times 10^6$	$1{\cdot}10 \times 10^6$	$< 6 \times 10^3$	0

* Suspensions aerated, transferred to test tube and incubated for 48 hours at 37° C.

at 37° C. was required before counting the plates towards the end of the investigation. The counts were also complicated by the appearance of variant colonies which were smaller and less pigmented than those originally growing. Organisms from these colonies showed no differences in either morphology or in coagulase activity from those of the original type of colony. This change was first noted with the suspension stored at 37° C., and later appeared in those stored at room temperature and at 20° C., but was not observed in that kept at 10° C. up to the end of the second month.

TABLE VII

VIABILITY AND EXTINCTION TIMES TO 1·0 PER CENT. AQUEOUS PHENOL SOLUTIONS OF WASHED SUSPENSIONS OF *Staphylococcus aureus* (N.C.T.C. 6571) STORED AT DIFFERENT TEMPERATURES

	10° C.		Room temperature (c. 18° C.)		20° C.		37° C.
Storage time (days)	Viable count/ml.	Extinction time (min.)	Viable count/ml.	Extinction time (min.)	Viable count/ml.	Extinction time (min.)	Viable count/ml.
0	$2·42 \times 10^9$	—	—	—	—	—	—
5	$1·78 \times 10^9$	—	—	—	$9·0 \times 10^8$	—	—
8	$1·10 \times 10^9$	10·8	—	—	$4·75 \times 10^8$	—	—
12	$5·40 \times 10^8$	—	$1·50 \times 10^8$	—	—	9·80	—
14	—	—	—	—	$2·04 \times 10^7$	—	—
15	—	—	—	6·78	—	—	—
16	—	—	—	—	—	—	—
20	$1·04 \times 10^8$	—	—	—	$1·04 \times 10^7$	—	—
29	—	—	$5·10 \times 10^7$	—	—	—	—
30	$3·66 \times 10^7$	5·95	—	—	—	—	—
34	—	—	—	—	$4·20 \times 10^7$	3·36	$2·66 \times 10^6$
48	—	—	$3·84 \times 10^7$	3·89	—	—	—
56	$1·32 \times 10^6$	—	—	—	$2·08 \times 10^8$	—	$1·34 \times 10^6$

Effect of change of storage temperature

Examination of Tables III and IV shows that both viability and extinction times of *Bact. coli* fall more rapidly on storage of suspensions at 37° C. than at the lower temperatures. After storage of the unwashed suspension for a little over one month, several ampoules were removed from the 37° C. incubator and kept at room temperature. After further varying periods, ampoules of changed and unchanged storage temperature were opened and extinction times on exposure to 0·7 per cent. phenol were measured. Two months later, a further sample of ampoules was similarly removed from the incubator and, in this case, both extinction times and viable counts were determined. The results given in Table VIII show that extinction times of suspensions of changed storage temperature do not fall far below the values determined at the time of removal. From the few counts which were performed, it appears that the viability of the changed temperature suspension does not decrease with storage to the same extent as with suspensions maintained at 37° C. throughout.

Growth in aqueous suspensions of Bacterium coli

Suspensions of unwashed cells, which had been stored at 37° C. and 10° C. for 2 months, were centrifuged, and the supernatant liquids separately filtered to remove bacteria. The filtered supernatant from

TABLE VIII

EFFECT OF CHANGE OF STORAGE TEMPERATURE ON VIABILITY AND EXTINCTION TIMES OF UNWASHED SUSPENSIONS OF *Bact. coli*

Age of suspension stored at 37° C.	Duration of continued storage at 37° C. (days)	Extinction time (minutes)	Viable count/ml.	Duration of continued storage at room temperature (days)	Extinction time (mins.)	Viable count/ml.
34 days	1	34·8	—	—	—	—
	8	20·0	—	8	32·2	—
	22	10·8	—	22	39·3	—
	35	6·63	—	36	37·4	—
57 days	1	10·8	—	—	—	—
	11	6·63	$2·55 \times 10^5$	13	8·26	—
	78	4·90	$1·05 \times 10^5$	81	8·47	$2·02 \times 10^5$
	117	5·63	$1·35 \times 10^5$	117	8·45	$2·09 \times 10^5$

the suspension formerly stored at 10° C. was then added to the packed cells of the suspension formerly stored at 37° C. Similarly, the filtered supernatant from the suspension previously stored at 37° C. was added to the packed cells of the suspension which had been stored at 10° C. The new suspensions were stored at the same temperature as that at which the cells they had originally contained had been stored. Viable counts and extinction times to phenol solutions were determined after a further 7 days. It was found that the treated suspensions stored at 10° C. had not undergone any appreciable change as compared with untreated suspensions. Treated suspensions stored at 37° C., however, had undergone a great increase in viability (from $2·55 \times 10^5$ to $2·04 \times 10^8$ per ml.) and in extinction time (from about 11 minutes on exposure to 0·7 per cent. phenol to 25·5 minutes with 1·05 per cent. phenol). Viable counts were also made at short intervals beginning immediately after transference of the supernatant; the results showed that the increase in viability was gradual. The plot of logarithms of bacterial numbers against time (Fig. 2, curve A) resembles a growth curve.

Ampoules of unwashed suspension which had been stored at 37° C., were opened, the liquid aerated by bubbling sterile air from a capillary pipette, and the suspension transferred to a sterile test tube. On re-incubation, the viable count increased to almost the same extent as described above and as shown in Figure 2, curve B. Removal of the supernatant from suspensions stored at 37° C. and replacement with distilled water led to nearly the same increase in viable count after re-incubation (Fig. 2, curve C).

The second row of values in Tables V and VI relate to aerated and re-incubated suspensions; they illustrate the effect of type of container, volume of suspension contained and presence of carbon dioxide on the increase in viability which ensues.

DISCUSSION

The preliminary results for the maintenance of viability and sensitivity to bacteriostatics of suspensions of several different organisms (Tables I and II, Fig. 1) show that more than 1 per cent. viability of all suspensions

was maintained for at least 2 months. A spore-forming organism, *Bacillus anthracis*, and an acid-fast organism, *Mycobacterium smegmatis*, underwent increases in viability over part of the period. The preliminary results, although for a different strain of *Bacterium coli* than that used by Cook and Steel[4], indicate that aqueous suspensions of this organism are not so stable as had originally been supposed. However, resistance to bacteriostatics is probably maintained more constantly with *Bact. coli* than with the other organisms used in this investigation and it was for this reason that *Bact. coli* was selected for more detailed study. It will be noticed from Table II that variations in resistance appear to develop more readily to crystal violet than to most other bacteriostatic substances which were examined.

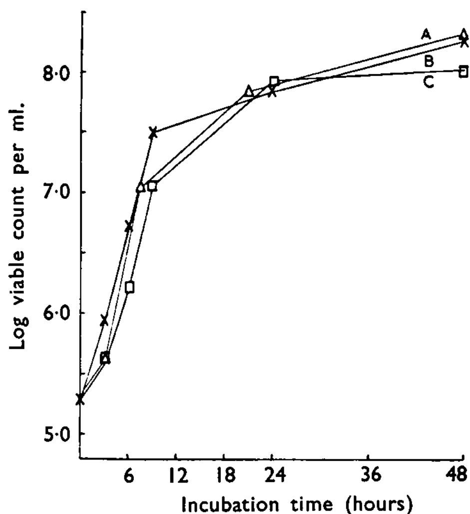

FIG. 2. Increase in viable count of suspensions of *Bacterium coli* stored at 37° C. for 2 months. Curve A (△——△) represents increases in viability when the cells of a suspension stored at 37° C. were resuspended in the supernatant of a suspension stored at 10° C. Curve B (×——×) was obtained on aeration and re-incubation of a suspension previously stored at 37° C. Curve C (□——□) represents the increase in viability when the cells of a suspension previously stored at 37° C. were resuspended in sterile distilled water.

Studies of the effects of temperature on the storage of unwashed suspensions of *Bact. coli* indicate that a greater stability is attained at temperatures at or below 10° C. The percentage survival and extinction times to 1·1 per cent. phenol solution (expressed as a per cent. of the original extinction time) after storage for 6 months were as follows.

°C.	Viability per cent.	Extinction time per cent.
4	13·9	41·5
10	3·47	50·0
Room temperature	0·53	30·2
20	0·31	14·8
37	0·007	Not comparable

The results were generally similar with the use of washed suspensions of *Bact. coli*, with the exception that a poorer survival is apparent at 4° C. Numbers of viable organisms per ml. of washed suspensions are shown plotted logarithmically against storage times at 3 separate storage temperatures in Figure 3. There is increase in slope with increase in temperature. The initial rate of fall in viability is not maintained for suspensions

stored at 37° C., and it is possible that, over a longer period of storage, the time-survival curves may be non-linear. The extinction times are lower than those obtained with unwashed suspensions of the same viability and it appears likely that thorough washing of the cells leads to a lower value of extinction time.

The values recorded in Table VII indicate that washed suspensions of *Staph. aureus* underwent a greater loss of viability and greater fall in extinction time than did those of *Bact. coli*. It would appear that temperature has a smaller influence on survival of staphylococci.

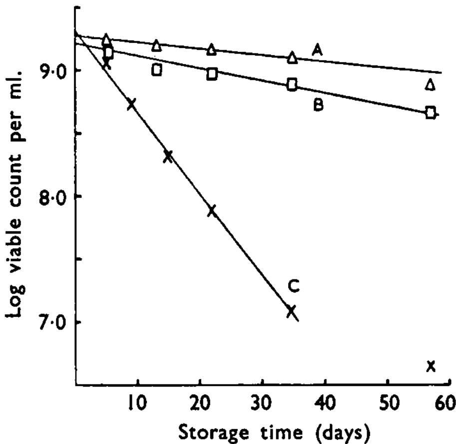

FIG. 3. Relationship between viability and storage time for the storage of washed suspensions of *Bacterium coli* at different temperatures. Curve A (△——△) stored at 10° C. Curve B (□——□) at 20° C. Curve C (×——×) at 37° C.

An indication of the most suitable containers for storage of suspensions of bacteria may be found from the values in Tables V and VI. Sealed ampoules seem to give lower survival than a bottle which is frequently opened to admit air. If it be assumed that the decay of suspensions at 37° C. constitutes only an acceleration of similar but much slower processes at lower temperatures, it may be concluded that suspensions may be stored in ampoules provided the volumes kept in the ampoules are small compared with the total capacity. The effects of type of container on the survival of *Staph. aureus* are much less certain.

The results throw some light on the possible mechanisms of survival of bacteria in water. It has been shown that *Bact. coli* surviving in the suspensions is able to increase in viability when suspended in its own ambient fluid, a filtrate of this fluid, or in distilled water. Viable counts after aeration and re-incubation (Table V) illustrate that the amount of regrowth is dependent upon the extent of decay of the suspension: the fewer the number of survivors, the greater the increase in viability which occurs afterwards. Where suspensions were stored at 37° C., decay was accelerated by storage under carbon dioxide, but the increase in viability after aeration and incubation was then the greatest. Stored in sealed ampoules under air, the rate of decay increased as the ampoule was more nearly filled and the increase in viability after aeration and re-incubation was found to increase correspondingly.

Two probable ways in which the increase in viability could be explained are (i) as a re-activation of cells presumed to be dead, or (ii) as actual growth of surviving bacteria. When the sterile supernatant from a

suspension was solidified with agar containing nutrients, there was no increase in the number of colonies from inocula of a stored suspension. This finding appears to contradict the hypothesis of re-activation. On the other hand, it was found that the increase in viability followed something very similar to a growth curve (Fig. 2). On the basis of all the evidence, it is considered that the process is probably one of growth, by which bacteria which die in the suspension are able to provide nutrient for use by the surviving organisms. That the process still took place when the cells were resuspended in water suggests that part at least of the nutrient is still associated with the dead cells and is not released into the suspension medium. Growth in such systems has been suggested by Steinhaus and Birkeland[11]. It will be noticed (Table VI) that a similar revival of *Staph. aureus* could not be demonstrated.

Survival of bacteria in stored suspensions can therefore be regarded as a dynamic process in which the survival of organisms is aided by those which have not survived. The survival rate is much decreased by either lack of oxygen or concentration of carbon dioxide and is dependent on temperature. Microscopical examination of stored suspensions did not show definite resting forms of *Bact. coli.* Cells were motile after several weeks but the cytoplasm appeared less dense than in freshly prepared suspensions.

It may be concluded that aqueous suspensions of bacteria may have application in testing bactericidal activity. *Bact. coli* appears to be a suitable organism for such application. If suspensions are stored at or below 20° C., they may be maintained for 1 month without appreciable decline in extinction times to aqueous phenol solutions; if stored at or below 10° C., suspensions may be kept for 2 or 3 months. If suspensions of constant viability are required, the period of safe storage is much shorter and probably does not exceed a few days at low temperature. The Oxford strain of *Staph. aureus* appears unsuitable for prolonged storage, but of the organisms employed in the preliminary investigation, *Salm. typhi* and other strains of *Staph. aureus* have a survival rate which is promising to the use of their stored suspensions in testing of disinfectants.

In a study of the factors which influence the reproducibility of extinction time estimates, Cook and Wills[3,12] concluded that variations among individual test suspensions was a major source of error. The use of stored suspensions may probably reduce this error, especially between different workers and laboratories. A method is provided by which extinction time determinations are made easier without sacrifice in accuracy.

Summary

1. The survival of several different bacterial species in aqueous suspensions stored at 10° C. and at room temperature has been compared.

2. Changes in sensitivity to bacteriostatics of a range of organisms in stored suspensions has been studied. *Bact. coli* probably suffered less change in sensitivity than the other organisms tested.

3. Changes in viability and extinction times to phenol of washed and unwashed suspensions of *Bact. coli* and of washed suspensions of *Staph.*

aureus (Oxford strain) have been measured. The suspensions were stored at different temperatures and in various containers. Loss of viability and fall in extinction times were roughly parallel, were dependent on storage temperature and influenced by type of container.

4. Stored suspensions of *Bact. coli* at 4° and 10° C. will give consistent extinction times to phenol over periods of 2 or 3 months. Changes in viability occur after short periods of storage, and if stored suspensions are required to have a constant viability they may be kept for no more than a few days. Decrease in viability and extinction times of *Staph. aureus* suspensions was much more rapid.

5. Possible mechanisms of survival of bacteria in aqueous suspensions have been discussed.

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DISCUSSION

The paper was presented by DR. B. A. WILLS.

The CHAIRMAN asked whether the authors considered that bacteria changed individually in their resistance; was there any evidence that some bacteria were dying and others replacing them? Had the authors carried out total counts to check whether there was an increase in the number of bacteria?

MR. J. A. VICKERS (Sunderland) pointed out that the organisms listed in Table I were counted by the Miles and Misra method, and asked whether any precautions were taken during the viable counting process to ensure that clumps of organisms were broken up.

DR. G. E. FOSTER (Dartford) said it seemed to him that there could not be more viable bacteria present than the number originally included.

MISS A. E. ROBINSON (London) said she noted that the survival of *E. coli* in stored suspensions was considered to be partly dependent on nutrients associated with the dead cells, and that this could not be shown in *Staph. aureus* suspensions. Was it possible that the chemical nature of the cell surface might account for this difference? Could the authors give further details of the standardisation of their bacterial suspensions since the optical density could be affected by a number of factors including the concentration of salts present.

DR. A. H. BECKETT (London) pointed out that as the authors had used only phenol as a bactericide it would be necessary to show that extinction times remained constant for each other bactericide examined before using the method, since bactericides exerted their effect by many different mechanisms.

DR. B. A. WILLS, in reply, said that a limited number of total counts of the bacteria in the suspensions was carried out, from which it appeared that the number of organisms remained approximately constant. The clumping of the organisms was not allowed for in the initial rough determinations which were made using suspensions of various organisms. The number of organisms did not increase in excess of the original number present. The number given was that originally present in the suspension as it was made. There might be an increase in the number present before reincubation was commenced but not beyond the number initially present. Due to the thorough washing of the suspensions they would remain unaffected by electrolyte concentration. He did not consider that the fact that the investigations had been restricted to one bactericide affected the validity of their conclusions. He agreed however that before one could start testing with other bactericides further work would be necessary using those bactericides.