THE RESISTANCES OF VEGETATIVE BACTERIA TO MOIST HEAT

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Washed suspensions of *Escherichia coli* required much shorter exposure to heating at 57° for sterilisation than unwashed suspensions, the greater resistance of which was due to materials carried over from the culture medium. The heat resistances of washed suspensions resuspended in solutions of electrolytes differed from those in non-electrolytes. The mode of protection is discussed in terms of the charge and permeability of the bacterial surface. Varying methods of culture and periods of growth markedly affected the heat resistances of washed suspensions. Aeration, exclusion of air, presence or absence of carbon dioxide and the surface moisture of solid media all influenced the heat resistance. Washed suspensions of *E. coli*, on storage, retained heat resistance poorly, in contrast to retention of viability and resistance to bactericides and bacteriostats.

Killing of bacteria by moist heat causes leakage from the cells of substances which will support growth of unexposed *E. coli*, but which will not support growth of survivors. Growth is also supported by killed cells themselves as distinct from substances leaking from the cells.

The killing of vegetative bacteria by exposure to moist heat and the alteration of heat resistance have received much attention since the early investigations of Chick[1]. The investigations of death rates of *Escherichia coli* at elevated temperatures carried out by Jordan, Jacobs and Davies[2] used constant food supply and aeration[3] and give little information of the behaviour if these conditions are not met. This paper presents results with washed suspensions of *E. coli* approximating to a broth culture in the stationary phase of growth.

Experimental

Bacterial suspensions. The organism was *Escherichia coli* Type I, a 44° positive laboratory strain, formerly N.C.T.C.5933. Suspensions were prepared from slope cultures[4,5], washed by three times centrifuging at 3000 r.p.m. for 25 minutes, resuspended in standardised volumes of sterile water, and finally adjusted to a density of 2×10^9/ml.

Materials. All chemical substances used were of A.R. purity with the exception of calcium chloride hydrate and glucose of Pharmacopoeial purity, "Oxoid" peptone, and Davis agar and gelatin. Nutrient media contained 1 per cent peptone, 0·5 per cent sodium chloride and were adjusted to pH 7·0. Solid media contained in addition 2 per cent Davis agar.

Methods. The suspension in 5 ml. volumes was sealed in sterile 5 ml. ampoules, weighted with small lead weights and immersed in a waterbath maintained at the desired temperature within limits of ±0·1°. After exposure, an ampoule was removed, cooled at room temperature for 15 minutes, thoroughly shaken, opened, and a sample diluted by a series of

tenfold dilutions with sterile water. Drops of these dilutions were placed with the Cook and Yousef[6] pipette on the surfaces of overdried plates to obtain viable counts of surviving organisms[7]. Plates were incubated at 37° for 12–15 hours and colonies counted from between 10 and 20 replicate platings. The following factors were found to be important:

The length of time the ampoules were allowed to stand at room temperature before dilution; a decrease in the numbers of surviving organisms was found if the ampoules were allowed to stand for more than 30 minutes. The count was approximately halved when suspensions heated at 57° for 1 hour were allowed to stand for 2½ hours.

TABLE I

NUMBERS OF SURVIVING VIABLE *E. coli* AFTER HEATING A WASHED SUSPENSION CONTAINING 2×10^9/ML. AT DIFFERENT TEMPERATURES AND TIMES

Time (minutes)	Viable count/ml. suspension — Temperature (° C.)			
	65	63·5	59	57
1	$4{\cdot}86 \times 10^7$			
3	$8{\cdot}11 \times 10^2$			
4	$7{\cdot}20 \times 10^1$	$1{\cdot}19 \times 10^6$		
5		$1{\cdot}04 \times 10^4$	$4{\cdot}91 \times 10^8$	
6		$2{\cdot}34 \times 10^3$		
10			$2{\cdot}38 \times 10^8$	
20			$7{\cdot}64 \times 10^5$	$1{\cdot}52 \times 10^9$
30			$2{\cdot}10 \times 10^2$	$6{\cdot}66 \times 10^8$
40				$1{\cdot}89 \times 10^8$
60				$1{\cdot}21 \times 10^6$
80				$9{\cdot}00 \times 10^1$

Delay in plating out the dilutions reduced the viable count.

Plates maintained at room temperature until the end of a series of experiments gave a lower count than those transferred to the incubator immediately after inoculation. Similar effects were not seen with unheated control suspensions. Organisms which survived the heat treatment may fail to reproduce unless rapidly transferred to an appropriate medium at a suitable growth temperature.

Statistical examination showed that the heterogeneity of colony counts among replicate platings generally increased as the mortality from heat treatment increased. Values of χ^2 corresponding to $P = 0{\cdot}05$ or over were obtained except where the number of colonies of survivors from the undiluted suspension was small.

To select a suitable temperature for future experiments, the numbers of viable organisms were counted after exposure of washed suspensions to varying temperatures for suitable times. Some results are listed in Table I. A temperature of 57° gave a substantial reduction of viability after 1 hour, and seemed convenient. Numbers of surviving organisms are shown plotted logarithmically against duration of heating for washed and unwashed suspensions at 57° in Figure 1. The curves are of similar sigmoid shape, the differences between the resistance of the suspensions increasing with exposure.

The lower resistance of the washed suspension may be due either to the removal of material derived from the medium (peptone, agar, salts) or to the removal of metabolites which exert a protective effect. When washed cells were resuspended in the condensate washed from several uninoculated agar slopes, the numbers of survivors approximated to those found with unwashed suspensions (Table II). Thus the first of the alternatives was likely to be true. Also increase in temperature during centrifugation and mechanical effects in precipitation and resuspension of the organisms were unlikely to affect heat resistance. Washed cells were next suspended in dilutions of a peptone water, 1 per cent peptone, 0·5 per cent sodium chloride, pH 7·0, when even 1 : 100 dilutions increased heat resistance. Finally, suspensions with varying concentrations of peptone were employed, without added electrolyte, in the suspending medium. The counts after heating at 57° for 1 hour are shown in Table III.

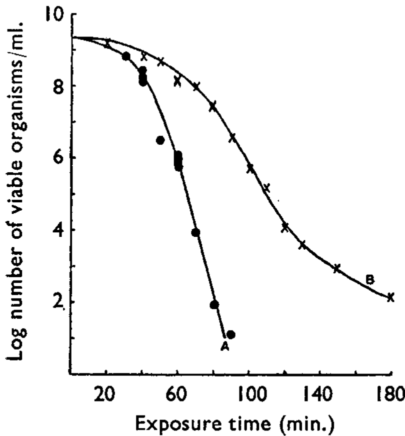

Fig. 1. The loss of viability of *Escherichia coli* when heated at 57°. A, washed suspensions; B, unwashed suspensions.

Since these peptone solutions were more acid than those previously used, washed cells were resuspended in 0·5 per cent peptone adjusted to 3 different hydrogen ion concentrations. The counts per ml. after heating for 1 hour were as follows: pH 5·5, $2{\cdot}44 \times 10^8$; pH 6·8, $2{\cdot}36 \times 10^8$; pH 8·6, $1{\cdot}28 \times 10^7$. It appears that small variations from neutrality influence the results only on the alkaline side.

TABLE II

EFFECT OF ADDITION OF SLOPE CONDENSATE TO WASHED SUSPENSION OF *E. coli* ON RESISTANCE TO HEATING AT 57°

Exposure time (min.)	Viable count/ml. suspension		
	Without slope condensate	With slope condensate	Unwashed suspension
50	$1{\cdot}77 \times 10^7$	$3{\cdot}56 \times 10^8$	$4{\cdot}81 \times 10^8$
80	$3{\cdot}00 \times 10^2$	$3{\cdot}62 \times 10^7$	$2{\cdot}26 \times 10^7$

In view of the common belief, cited by McCulloch[8] and Fay[9], that proteins and other colloidal materials are able to protect bacteria against killing by moist heat, solutions of agar and gelatin were examined. Both of these substances in low concentration were found to increase the heat resistance of washed cells (Table IV). If these substances and peptone exerted a protective action by virtue of their colloidal nature, a loss of protection should result from degradation by hydrolysis. Weighed

quantities of the three substances were dissolved in 4N sulphuric acid and autoclaved in glass-stoppered tubes for 8 hours at 121°. The cooled solutions were neutralised with chalk to avoid a high sulphate concentration, and were boiled and filtered. The cooled filtrates were adjusted to volume, sterilised by autoclaving and the washed organisms suspended in

TABLE III

EFFECT OF ADDITION OF VARYING CONCENTRATIONS OF PEPTONE ON HEAT RESISTANCE OF *E. coli* TO 57° FOR 60 MINUTES

Peptone concentration (per cent w/v)	Viable count per ml.	Peptone concentration (per cent w/v)	Viable count per ml.
0	$8{\cdot}32 \times 10^5$, $5{\cdot}22 \times 10^5$	0·10	$7{\cdot}82 \times 10^7$
0·005	$1{\cdot}40 \times 10^6$	0·15	$1{\cdot}08 \times 10^8$
0·025	$4{\cdot}86 \times 10^6$	0·20	$1{\cdot}39 \times 10^8$
0·050	$6{\cdot}12 \times 10^6$, $7{\cdot}48 \times 10^6$	0·30	$1{\cdot}44 \times 10^8$
0·075	$1{\cdot}60 \times 10^7$	0·50	$2{\cdot}18 \times 10^8$

these solutions. Results of survivor counts are shown in Table IV, together with the results from a "blank" solution prepared without the three substances. Increased resistance to the heat treatment was found with all three hydrolysates, but the "blank" solution and a separately prepared saturated aqueous solution of calcium sulphate had about an equal effect. The effects of the hydrolysis were apparently obscured by the protection afforded by salts present in the original substances and derived from the reagents.

TABLE IV

EFFECTS OF ADDITION OF PEPTONE, GELATIN, AGAR AND THEIR HYDROLYSATES ON RESISTANCE OF *E. coli* TO HEATING AT 57° FOR 60 MINUTES

Substance	Concentration (per cent w/v)	Viable count per ml.	
		Not hydrolysed	Hydrolysate
Peptone	0·5	$2{\cdot}12 \times 10^8$	$3{\cdot}40 \times 10^8$
Gelatin	0·05	$1{\cdot}04 \times 10^8$	$2{\cdot}10 \times 10^8$
Agar	0·05	$8{\cdot}28 \times 10^7$	$4{\cdot}44 \times 10^7$
	0·005	$5{\cdot}22 \times 10^6$	—
Blank	—	$2{\cdot}42 \times 10^8$	
Calcium sulphate	Saturated (20°)	$4{\cdot}17 \times 10^8$	

Effects of Electrolytes on Heat Resistance

Washed cells were resuspended in sterilised solutions of sodium chloride, calcium chloride, magnesium sulphate and sodium sulphate. The numbers of survivors after heating for 1 hour at 57° are shown plotted logarithmically against molar salt concentration in Figure 2. Even dilute solutions of all the salts had a protective action, but solutions more dilute than 0·01M were not examined; attention was given to the stronger solutions.

At the salt concentrations associated with minimal resistance (0·2 to 0·4M sodium chloride and about 0·2M of the other salts), growth of

colonies of the surviving organisms was so much slower than at either lower or higher concentrations that incubation for 18–24 hours was required before colonies could be counted. Anomalous counts were obtained from heated suspensions of sodium chloride in excess of 1·5M: drops of the undiluted suspension gave an uncountable number of colonies, whereas the first tenfold dilution gave few colonies and the successive dilutions were sterile. This was explained by plasmoptysis of the cells on transference to a medium of lower osmotic pressure in the first dilution. When the sodium chloride concentration of dilution fluid was the same as the suspension medium, normal counts were obtained.

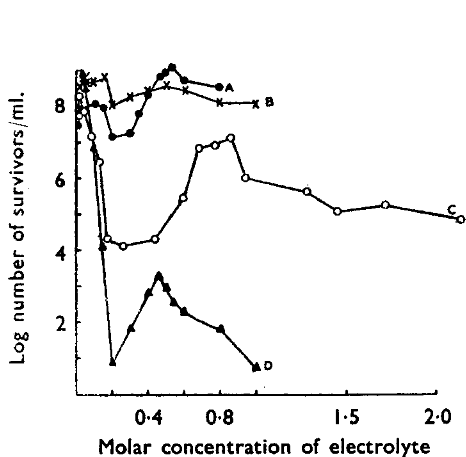

FIG. 2. The effects of electrolytes on the heat resistance of washed suspensions of *E. coli*. Plotted values are logarithms of numbers of organisms/ml. surviving exposure for one hour at 57°, at different molar salt concentrations. A, sodium sulphate; B, magnesium sulphate; C, sodium chloride; D, calcium chloride.

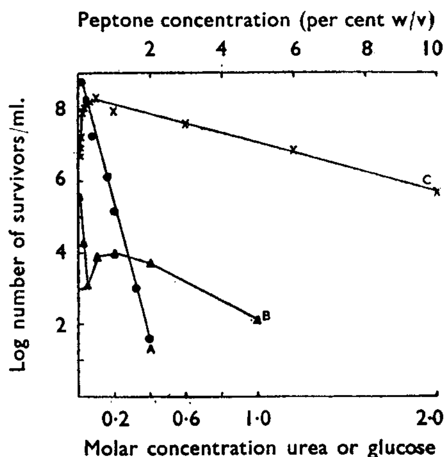

FIG. 3. The effects of organic compounds on the heat resistance of washed suspensions of *E. coli*. Logarithms of numbers of organisms/ml. surviving exposure for one hour at 57° in the presence of different concentrations of A, urea, B, glucose, and C, peptone.

The Effects of Other Substances

For comparison with the electrolytes used, the effects of non-ionisable substances were investigated. Results obtained when solutions of urea and glucose were used as suspension media are shown in Figure 3. Decreasing resistance with increasing urea concentration was very steep, very few surviving organisms remaining when 0·4M was used. In concentrations of 0·6, 1·0 and 2·0M urea no surviving bacteria were detected, even when the remainder of the contents of the ampoule was added to 200 ml. of a liquid medium, known to support growth of very small inocula of *E. coli* and incubated for a long period. The examination of peptone was extended to more concentrated solutions, and the survivor counts are plotted logarithmically against concentration per cent w/v in Figure 3.

Alterations of Heat Resistance with the Use of Different Cultural Conditions

Suspensions were prepared from 24 hour surface growth of *E. coli* on overdried and undried agar plates; overdried plates incubated in McKintosh and Fildes' jars with widely differing carbon dioxide contents, overdried plates filled to different depths with agar and sealed with cellulose tape, and sealed plates the lids of which were filled with soda lime. Survivor counts obtained after the heat treatment are recorded in Table V.

TABLE V

Numbers of *E. coli* surviving exposure to 57° for 60 minutes, the washed suspensions derived from different cultures

Culture	Cultural conditions	Age (hours)	Viable count per ml. Before treatment	Viable count per ml. After treatment
Undried plate	20 ml., unsealed	24	$1{\cdot}99 \times 10^{9}$	$4{\cdot}90 \times 10^{8}$
Overdried plate	20 ml., unsealed	24	$1{\cdot}97 \times 10^{9}$	$5{\cdot}88 \times 10^{6}$
	10 ml., sealed	24	$2{\cdot}10 \times 10^{9}$	$1{\cdot}99 \times 10^{5}$
	40 ml., sealed	24	$2{\cdot}01 \times 10^{9}$	$2{\cdot}60 \times 10^{4}$
	20 ml., sealed, approx. $\frac{1}{2}$ CO_2	24	—	$1{\cdot}48 \times 10^{4}$
	20 ml., sealed, approx. $\frac{3}{4}$ CO_2	24	$1{\cdot}91 \times 10^{9}$	$3{\cdot}46 \times 10^{3}$
	20 ml., sealed, almost solely CO_2	24	$1{\cdot}69 \times 10^{9}$	$1{\cdot}68 \times 10^{3}$
	20 ml., sealed, over soda lime	24	$1{\cdot}74 \times 10^{9}$	$2{\cdot}33 \times 10^{3}$
	20 ml., sealed, over soda lime	48*	$1{\cdot}50 \times 10^{8}$	0
Peptone water	Not aerated, not agitated	24	$1{\cdot}73 \times 10^{9}$	$1{\cdot}10 \times 10^{6}$
		48	$1{\cdot}78 \times 10^{9}$	$8{\cdot}68 \times 10^{4}$

* Strong odour of ammonia on opening plate.

Washed suspensions were also obtained from the cells centrifuged from cultures in peptone water. The cultures were either aerated at a rate rapid enough to justify saturation with oxygen or were not aerated or agitated. All cultures were incubated at 37°. Figure 4 shows the logarithms of survivor counts after heating for 1 hour at 57° plotted against duration of incubation of aerated fluid cultures (curve B), the initial viability of each suspension expressed as a percentage of the viability of a suspension prepared from a 12 hour culture (curve C), and the viable counts of the fluid culture (curve A). Heat resistance of cells from the youngest culture (12 hours) is low and rapidly increases with age in accordance with the findings of Chick[1] and Elliker and Frazier[10], who used non-aerated cultures. The increase in resistance coincides with a sharp fall in the viability of the suspensions (curve C). A fall in suspension viability may mean a reduction in viability of the culture; but, since the suspensions were adjusted densiometrically by measuring the total bacterial substance present, it may also mean an increase in average cell size. Maximal resistance was achieved after growth for about 24 hours.

Longer incubation decreased heat resistance, the lowest resistance being met after about 35 hours, when a further increase was apparent, although subject to doubt in view of wider discrepancies between results from duplicate cultures than had hitherto been encountered. The sharp decline in heat resistance after reaching a maximal resistance does not appear so far to have been reported, and may be explained by the use of

washed suspensions and aerated culture. Aerated cultures achieve a stationary population much more rapidly than static cultures (curves A and D, Fig. 4), and suspensions from static cultures gave a more constant resistance with varying incubation time of the culture (Table V).

Heat Resistance of Stored Suspensions

In view of the findings of Cook, Steel and Wills[11] on the maintenance of resistance of stored bacterial suspensions to bactericides and bacteriostats, washed suspensions of *E. coli* were stored at room temperature in glass-stoppered bottles and subjected to heat treatment after varying periods. Some results are listed in Table VI. Suspensions were adjusted to twice the usual density, i.e. 4×10^9/ml., and were diluted with an equal volume of either water or a 1 per cent w/v peptone solution before exposure to heat. Loss of heat resistance in stored suspensions is rapid, particularly within the first 48 hours, but suspension in 0·5 per cent peptone gives protection during the first few days only.

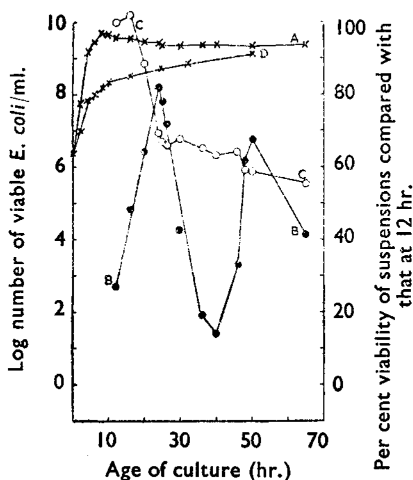

FIG. 4. The effects of age of culture of *E. coli* on the viability and heat resistance of washed suspensions. Suspensions were prepared from aerated broth cultures. A relates the viable count/ml. of broth culture to duration of incubation; C was obtained by plotting the per cent viability of washed suspensions compared with that of a suspension prepared from a 12 hour culture; B refers to the surviving organisms after heating the washed suspensions for one hour at 57°; D shows the viability of an unaerated, static broth culture after varying periods of incubation.

Growth-supporting Materials from Heat-killed Organisms

Previously[11] *E. coli* has been found to increase in viability when the cells from a suspension stored at 37° for 2 months were resuspended either in an aerated suspension supernatant, or in water. It was concluded that the organisms which had survived storage were able to grow on substances provided by dead cells. Since amino acids leak from vegetative bacteria when killed by moist heat[12], whether heat-killed cells could support bacterial growth was investigated.

A washed, living suspension of *E. coli* was prepared, a portion reserved for inoculation of the killed preparations, and the remainder filled into ampoules and heated at 65° for 45 minutes, which results in sterilisation. The cells were centrifuged, resuspended in water, and this washing repeated 5 times. The first and second washings were reserved and separately filtered through sintered glass bacterial filters. 5 ml. portions of each filtrate were inoculated with one drop of living *E. coli* suspension

so diluted that a count of about 1×10^5/ml. resulted. The determined initial viable count was $1{\cdot}51 \times 10^5$/ml. Counts per ml. after incubation for 24 hours were as follows:

In the first washings, $1{\cdot}63 \times 10^7$, and in the second, $1{\cdot}21 \times 10^6$.

In the first washings, after boiling 1 minute and cooling, $1{\cdot}71 \times 10^7$. Inoculation of 5 ml. sterile distilled water and incubation gave a count of $9{\cdot}00 \times 10^4$/ml. The first and second washings of the living suspension gave counts after inoculation and incubation of $1{\cdot}40 \times 10^8$ and $2{\cdot}57 \times 10^6$/ml. respectively, so that little growth would be expected from a third washing.

TABLE VI

NUMBERS OF VIABLE *E. coli* PER ML. SURVIVING EXPOSURE TO 57° FOR 60 MINUTES IN STORED WASHED SUSPENSIONS

Culture	Medium	Age of suspension (days)						
		0	1	2	5	7	14	28
Slope	Water 0·5 per cent peptone	$6{\cdot}90 \times 10^5$ $1{\cdot}21 \times 10^8$	$1{\cdot}51 \times 10^5$ $6{\cdot}72 \times 10^7$	$8{\cdot}28 \times 10^3$ $3{\cdot}57 \times 10^7$	$6{\cdot}12 \times 10^3$ $6{\cdot}66 \times 10^6$	$1{\cdot}17 \times 10^4$ $9{\cdot}02 \times 10^6$	$1{\cdot}11 \times 10^3$ $8{\cdot}40 \times 10^2$	$2{\cdot}04 \times 10$ $1{\cdot}44 \times 10^2$
Aerated broth (24 hr.)	Water	$1{\cdot}53 \times 10^6$	$2{\cdot}24 \times 10^3$	—	—	0	—	—
Unaerated broth (24 hr.)	Water	$1{\cdot}10 \times 10^6$	$2{\cdot}53 \times 10^5$	—	$4{\cdot}47 \times 10^3$	—	—	—
Overdried plate	Water	$5{\cdot}88 \times 10^6$	$6{\cdot}18 \times 10^5$	$8{\cdot}90 \times 10^3$	—	—	—	—

The results indicate that killing of suspensions of *E. coli* under the above conditions is accompanied by a loss of growth-promoting material to the suspension medium. Such materials are stable to boiling, and support growth, but not so well as the first washing in preparation of the living suspension, which contains nutrients removed from the agar slope.

The washed, killed cells from the above experiment were suspended in water, adjusted to a density of 2×10^9/ml., and placed in 10 ml. volumes in capped tubes which were incubated at 37°. After incubation for 27 days, half of the suspension was filtered to remove bacteria and the filtrate and the unfiltered suspension inoculated with freshly prepared suspension of *E. coli.* On incubation, the following viable counts per ml. were obtained.

Incubation time, hr.	0	16	48
Count in filtrate	$1{\cdot}96 \times 10^5$	$1{\cdot}61 \times 10^5$	$4{\cdot}74 \times 10^4$
Count in suspension ..	$2{\cdot}18 \times 10^5$	$1{\cdot}78 \times 10^7$	$2{\cdot}51 \times 10^7$

It appears that the prolonged storage of a thoroughly-washed suspension of heat-killed organisms in water results in the release of little more of the growth materials that occurred during the killing process. The system of cells + suspending fluid, however, is able to support growth, a finding which supports previous hypotheses[11,13] that bacterial cells

themselves, apart from products of metabolism and death, may provide nutrient for growth of living cells.

That a small proportion of organisms surviving a heat treatment might be able to grow using materials derived from the dead cells was investigated. Washed suspensions were heated for various times at 57°, 59° and 65° and viable counts of survivors determined. The suspensions were then incubated at 37° for 24 and 48 hours and the counts again estimated. There was no definite increase in viability after incubation. In fact, where the numbers of surviving organisms were initially small (less than 0·1 per cent survival), incubation usually showed a decrease in viability. Either the organisms surviving the heat treatment suffered damage which prevented their growth under conditions in which unheated cells can grow, or the milder conditions employed in these experiments caused insufficient damage to or leakage of contents from the killed cells to support growth.

Similar results have since been reported by Chambers, Tabak and Kabler[14], who mixed the treated suspensions of *E. coli* with equal volumes of 0·1M phosphate buffer solution or of solutions of Krebs cycle metabolites in phosphate buffer before incubation. The same workers substantiated the findings of Garvie[15] that organisms surviving treatment with chlorine, increased in viability after inactivation of the chlorine and transference to the above solutions. They considered that the increase in viability was due rather to multiplication of survivors than to a "reactivation" of presumed killed cells as postulated by Heinmets and others[16] Our evidence of growth of *E. coli* in stored suspensions[11] and of growth supported by killed cells suggests, contrary to Garvie's findings, that killed cells might easily be a source of nutrient in growth after chlorine treatment.

Discussion

Washed suspensions of *E. coli* require a considerably shorter time for virtual sterilisation than unwashed suspensions. The substances which confer resistance to unwashed suspensions are those carried over from the culture medium. This is suggested by the finding that peptone, agar and sodium chloride in low concentration are all able to confer similar resistance to washed cells. Peptone and agar may not act by the colloidal properties of their solutions but possibly because they contain ionisable compounds. Both substances contain inorganic salts.

The effects of added salts, in varying concentrations, on the heat resistance of washed suspensions show differences in the toxicity of the different ions. Calcium is much more toxic than magnesium or sodium; chloride is more toxic than sulphate. High toxicity of calcium salts to *E. coli* in suspensions at room temperature has been recorded by Winslow and Falk[17] and explained by the degree of dissociation of calcium salts by Mitchell[18]. Greater effect in preventing growth of several micro-organisms in liquid media by Ca^{++} than by Mg^{++} or Na^{+} has been described by several workers[17,19,20] and under similar conditions SO_4^{-} has been alleged[20] to be less toxic than Cl^{-}. Winslow and Falk found a minimal rate of

loss of viability of washed suspensions of *E. coli* at a sodium chloride concentration of about 0·25M at pH 7·0 and room temperature. This, however, is within the concentration range of maximal loss of viability at 57° (Fig. 2). Thus the salt appears to exert different effects at different temperatures. Winslow and Falk concluded that the salt acted mainly in an osmotic capacity because other univalent electrolytes behaved in the same way.

Effect of varying the concentration of electrolyte (Fig. 2) reveals a similar behaviour with all the salts examined. Variation in heat resistance at different concentrations of magnesium sulphate were ill-defined, but not with the other salts. Very low salt concentrations cause an increased resistance; increasing concentrations result in loss of resistance until a concentration of minimal resistance is reached (0·2–0·4M sodium chloride, 0·2M of the other salts). Further increases in concentration lead to increased resistance, which reaches a maximal value at a concentration of about 0·9M sodium chloride and 0·45–0·5M of the other salts. When the concentration is still further increased there is a drop in resistance, the rate of which depends upon the toxicity of the salt.

Previous reports of the effects of salts on heat resistance are vague. Von Angerer and Küster[21] found that solutions of calcium chloride offered no protection to *E. coli*; Robertson[22] reported a decrease in resistance with "hypotonic" salt solutions; Rahn[23] attributed the effects of salts to their osmotic pressures. My results offer no simple explanation in terms of osmotic effect. A tentative explanation is advanced on the following lines. Considerable evidence has led to the suggestion[18] that damage to the osmotic barrier of bacteria may result from thermal damage. The maintenance of the functions of the cell surface will depend *inter alia* on the surface charge, which has been shown[24,25] to be negative at physiological pH. In small concentrations, salts of univalent cations are held[26] to increase the surface potential; with increasing concentrations, cations decrease the potential, although univalent and divalent cations are considered incapable of neutralisation and reversal of the charge on the surface. In this case, only the initial increase and succeeding decrease in resistance can be explained by respective charging and discharging by electrolyte; and the second increase in resistance at higher concentrations might be explained as an osmotic effect whereby the loss of cellular constituents is retarded. The bacteria, at the salt concentration of minimal resistance, may have a nearly neutralised surface potential, making them more susceptible to heat and recovery of survivors more lengthy.

In contrast with electrolytes, increasing concentrations of urea caused a sharp decline in heat resistance (Fig. 3) without giving protection at any but very low concentrations. Suspension of washed cells in glucose solutions had decreased heat resistance at all concentrations, except for a slight increase at 0·05–0·1M. Differences in the behaviour of solutions of urea and glucose might be expected. Sterilised solutions of urea were alkaline (1M solution of pH 9·0), whereas those of glucose were faintly acid (1M solution of pH 6·2). Loss of resistance was found to be smaller when the glucose (0·02M) was dissolved in a phosphate buffer solution

(pH 7·0), but the resistance was then lower than with the use of phosphate buffer alone. Also, urea is a small molecule with high permeability of the lipoid cell membrane[27], and probably of lower osmotic effect than glucose; moreover, a high penetration of the cell by urea may occur, with resulting toxicity.

Previous workers have pointed to protection of bacteria against heat in the presence of sugars. Rahn[23] considered that increased resistance in the presence of strong sugar solutions was generally accepted and was explained by a partial dehydration of the protoplasm; Von Angerer and Küster[21] found that starch and urea gave no protection to *E. coli* heated at 56°, but inclusion of glycerol, sorbitol, monosaccharides and disaccharides retarded death. Previous investigations of the effects of added substances are subject to the general criticisms that the effects of change of concentration have been inadequately studied. Also attempts have rarely been made to remove substances carried over from the culture medium which greatly affect the response to heat.

In conclusion, it is found that a difference exists between the effects of electrolytes and non-electrolytes on the resistance of bacteria to moist heat and hence it is suggested between their modes of protection. In the protection of bacteria against heat, added substances act not in an osmotic capacity alone: presence of ions, permeability to the cell membrane and inherent toxicity of the substance are of importance.

The estimation of survival of washed bacteria when grown under different cultural conditions illustrates the very wide observed fluctuations in heat resistance. The following general conclusions are put forward. Aeration during growth of a culture leads to increased resistance when the culture is 20–25 hours old. Exclusion of air or the presence of high carbon dioxide contents in the atmosphere of growths results in lowered resistance. Complete absence of carbon dioxide and absorption of the gas liberated during growth causes reduced heat resistance, especially on long incubation. When grown on a solid medium, a moist surface appears to result in higher heat resistance.

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DISCUSSION

The paper was presented by the AUTHOR.

The CHAIRMAN. The surprising and interesting point was that growth could be supported by the killed cells themselves and not by metabolites or materials which had leaked out of those cells.

MR. K. A. LEES (London). The title was not sufficiently specific in that the paper dealt with one strain of one species only. The last paragraph of the Discussion referred to general conclusions, but if the work were repeated with other organisms it was doubtful if they applied.

DR. J. C. PARKINSON (Brighton) asked whether with glucose and urea the effect on lowered heat resistance was the effect of those substances or one of pH.

MR. H. D. RAPSON (Dorking). If some of the ions in a solution were fixed on the surface of the cell and the pH were changed the charge on the cell was also changed. Was the author able to determine the point at which the surface appeared to be of the same potential as the solution surrounding it? What was meant by the term "osmotic effect"?

MISS A. E. ROBINSON (London). Were the conclusions based upon the results at 57° significant at other temperatures? Reversal of charge had been demonstrated with certain divalent cations. The values for the internal osmotic pressure of *E. coli* quoted in the literature varied between 2 and 15–20 atmospheres. Had the author information about his strain of organism? Damage to the osmotic barrier for low molecular weight substances was a consequence of thermal damage to the bacteria.

MR. G. SYKES (Nottingham). If, after heating, the suspension were allowed to stand for 2½ hours instead of 1 hour, the result of the test was varied. The same applied if the plates were allowed to stand for a long interval. Was it a question of changes of temperature, and would it apply if a liquid culture had been used instead of a solid one? The work underlined the unreliability of any test which applied a so-called 100 per cent kill. The 90 per cent survival limit was no better from the point of view of reproducibility than the 100 per cent limit. Would the author comment on the work of Berry and his colleagues that a six-fold replicate of a 100 per cent endpoint was perfectly reproducible?

DR. WILLS replied he had intended to convey general conclusions based on his results rather than general conclusions for all organisms. In the use of urea the pH had a large bearing on the result. He had not made any measurements of surface neutrality. By osmotic effect he had in mind a greater concentration of solute in the external environment of the cells which would prevent leakage of constituents from the cell. He had done nothing to identify the substances which leaked from the cells. Behaviour at other temperatures had not been investigated. It was a fact that thermal death might be ascribed to damage to the osmotic barrier. By careful control of a number of factors like the medium and the conditions of growth, results could be obtained by Berry's method which were of very much closer reproducibility than would have been the case by any other bactericidal evaluation. The kill was not 100 per cent by this method but it was very close. He defended extinction methods because he had found such methods reproducible in his own hands, and because they more nearly approached the conditions in which one was interested in disinfection.