STUDIES IN THE DYNAMICS OF DISINFECTION

XI. THE EFFECT OF LETHAL TEMPERATURES ON STANDARD CULTURES OF *BACT. COLI*. IV. AN INVESTIGATION OF THAT PORTION OF THE POPULATION WHICH SURVIVES PROLONGED EXPOSURE AT *p*H 7·0

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(With 6 Figures in the Text)

It is natural to expect that in a bacterial culture exposed to a lethal temperature there should be a small number of viable cells remaining long after the majority have been killed, because the destruction of a culture is not an instantaneous but a progressive reaction. Beamer & Tanner (1939) showed that there was a diminution in the logarithmic death-rate in cultures of certain *Salmonella* species heated in broth at temperatures of 55–60° C. after the reaction had gone a long way (99·99 % or more) towards completion. This diminution in death-rate led to a still more prolonged survival of some of the remaining 0·01 % of cells, but it appeared that death was still occurring continuously. Jordan, Jacobs & Davies (1947), working with *Bact. coli* cultures heated to temperatures ranging from 47 to 53° C., found that after the mortality had reached approximately 99·99 % there was a sudden cessation of the killing process. Subsequently residual low populations of viable cells persisted for as long as the experiments were continued and would apparently have persisted indefinitely. The numbers of these residual cells fluctuated widely for some reason unknown, but there was no doubt of the continued existence of viable organisms. These cells evidently differed markedly from the majority in the cultures and may obviously be of considerable practical importance, for example, in the pasteurization of milk.

There is an interesting problem in the metabolic condition of these cells which remain alive at relatively high temperatures. They may be in a dormant state, or metabolizing but incapable of division, or possibly even passing through cycles of growth and division followed by death, leading to fluctuations in numbers without the attainment of large populations. Whatever the condition of the cells, their origin is equally interesting. They may have arisen through adaptation occurring during exposure to the high temperature or, alternatively, they may constitute the more highly resistant individuals in populations with wide ranges of susceptibility to heat, the distribution of susceptibility governing the shape of the mortality curve. An attempt has been made to discover which of these two possibilities is the more likely by allowing the permanent survivors from standard cultures of *Bact. coli* which have been subjected to lethal temperatures to generate fresh cultures, the heat resistance of which was in turn examined, and the results are reported below.

METHODS

Although the apparatus and methods employed for producing and maintaining the standard cultures of *Bact. coli* upon which the experiments described here were performed have been described in the first paper of this series (Jordan & Jacobs, 1944*a*), it seems desirable to recapitulate briefly the salient features of the technique, which differs considerably from that usually employed in disinfection studies. The reason for this recapitulation is that without a clear picture of the special features of the technique the behaviour of the cultures here described cannot be properly understood.

The standard culture was grown at 35° C. in the presence of *M*/30 phosphate buffer at *p*H 7·0, under continuous aeration and with accurate temperature control, in a 5 l. pyrex culture flask immersed in a thermostatically controlled water-bath. The 1450 ml. of medium contained, before inoculation, 1 g. of Difco dehydrated broth, but during the growth of the culture and throughout the disinfection process a food solution containing 6·0 g. of Difco dehydrated broth per litre (before autoclaving) was added at the rate of 0·066 ml./100 sec.

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This was equivalent to an actual addition of 15·2 mg. of the dehydrated broth per hr. The automatic syringe mechanism of Sims & Jordan (1941) was utilized to effect the food addition.

The continuous aeration referred to above served to distribute the food equally throughout the culture, to maintain uniformity and prevent sedimentation, to establish gaseous equilibrium and to ensure that shortage of oxygen should not limit growth and possibly lead to considerable differences between replicate cultures. By arranging the apparatus so that the ingoing air stream surrounded the food jet, contamination of the latter, and from thence of the food-solution reservoir, by bacteria-laden spray from the culture was prevented, and on this precaution the success of the technique depended. The air stream was dried and warmed to the temperature of the culture before it entered the flask, and the rate of flow was adjusted so that the increase in the volume of the culture resulting from the addition of the food solution was compensated by the evaporation of water by the dry air.

Under these conditions of controlled *p*H, aeration and food supply at 35° C., the viable population of the culture rose within a few hours to approximately 330 million cells per ml., at which level the viable count remained constant as long as the food addition was continued, as shown by Jordan & Jacobs (1944*b*). The population level was in fact controlled by the rate of food addition. This culture could be readily reproduced and constituted the standard culture for each disinfection experiment. A period of 40 hr. from the time of inoculation was in all cases allowed for stabilization of the culture before the disinfection was commenced.

In the experiments described below the standard cultures were exposed to lethal temperatures and the course of the disinfections followed by making viable counts on samples taken at convenient intervals. The method adopted for rapidly raising the temperature of the cultures to the desired level has already been described (Jordan *et al.* 1947). As soon as the lethal temperature had been attained and death of the cells had commenced, the total cell metabolism must have been greatly reduced and food must therefore have accumulated. After the active phase of disinfection had been completed and the permanent surviving population established for a considerable time, a fresh culture was generated in the same flask from these survivors by merely cooling the culture to 35° C. again. Hot water was siphoned out of the bath and replaced by cold so as to bring the culture temperature down rapidly, the thermostat being readjusted to the lower (growth) temperature. The new culture developed by making use of the food which had accumulated during the heat treatment of the primary culture and that which was being added throughout the regeneration period. No food addition other than the regular automatic one was made in these experiments except in one special case referred to below.

EXPERIMENTAL

One way in which the origin and nature of these permanently heat-resistant *Bact. coli* cells can be investigated is to allow them to grow into a new culture at a suitable temperature and to subject this in turn to heat disinfection. If the cells were able to transmit their resistance to their progeny, the regenerated culture should show an enhanced heat resistance and also a larger proportion of permanently surviving cells. In the absence of such transmission the new culture should behave in the same way as the original, provided that the milieu remained unchanged. Experiments were therefore carried out at 49 and 53° C. in which, after disinfection of the primary culture and the establishment of the permanent population, the temperature was reduced to 35° C. and the surviving cells allowed to develop into a new culture. This in turn was subjected to the lethal temperature as before and a further permanent population established. The viable counts throughout these experiments are given in Tables 1 and 2. As the experiments were very prolonged, lasting for 220 and 400 hr. at 53 and 49° C. respectively, it is impossible to present the complete results of each experiment adequately on one graph. It is clear, however, from the tables that the temperature reduction resulted in a prompt regeneration of the culture from the few cells surviving the first heat treatment, and it is considered unnecessary to present the regeneration phases graphically. Accordingly, in Figs. 1 and 2, where the logarithms of the numbers of survivors per ml. are plotted against time, only the periods of heat treatment are represented. At each lethal temperature it is clear that the disinfection of the regenerated culture proceeded much more slowly than did that of the primary culture.

In a previous paper (Jordan *et al.* 1947) it was shown that between the approximate limits of 10 and 0·01 % survivors there was a linear relationship between log survivors and time in the primary culture. The disinfection curves of the regenerated cultures can be treated in the same way (irrespective of whether this is the best treatment), and Table 3 gives a comparison of the slopes of the lines of regression of log survivors on time for the two types of culture at both lethal temperatures. The values of these slopes are actually the maximum death-rates for the cultures. The straight portions of the graphs in Figs. 1 and 2 have the slopes set out in Table 3, and the maximum death-rate in each case is seen to be smaller in the regenerated than in the primary culture. This would appear to be evidence

of an increased heat resistance following regeneration. However, another measure of the heat resistance of a culture is the disinfection time, previously defined for these cultures (Jordan *et al.* 1947) as the 99·99 % mortality time, which was obtained by calculation from the regression of log survivors on time. These times are also given in Table 3, and clearly the disinfection times were considerably apparent that the earlier stages of the disinfection curves for the regenerated cultures lie at a higher level than those of the primary cultures. The increase in disinfection time following regeneration is evidently attributable in large part to a decreased death-rate during the early phase of the mortality, and the effect of regeneration was thus reflected in an enhanced resistance of the population as a whole,

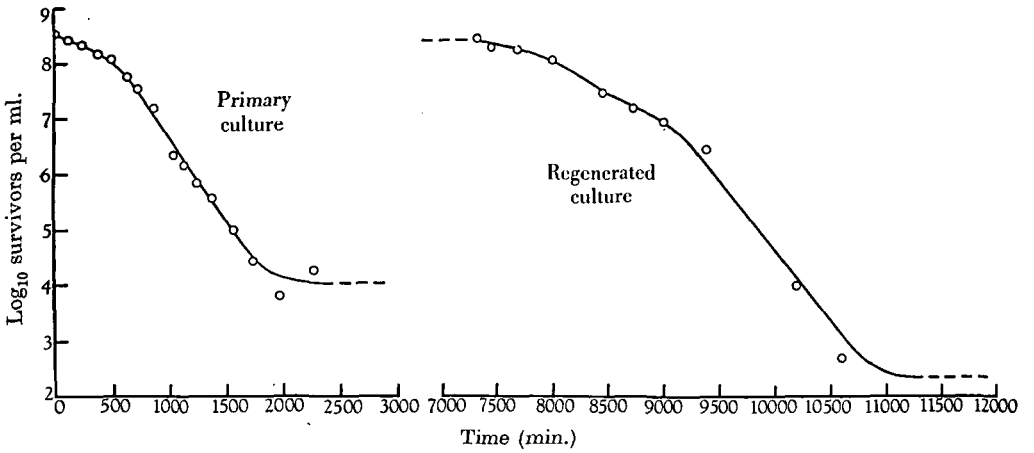

Fig. 1. Showing the relationship between logarithm of survivors and time for the primary and regenerated cultures of *Bact. coli* exposed to 49° C.

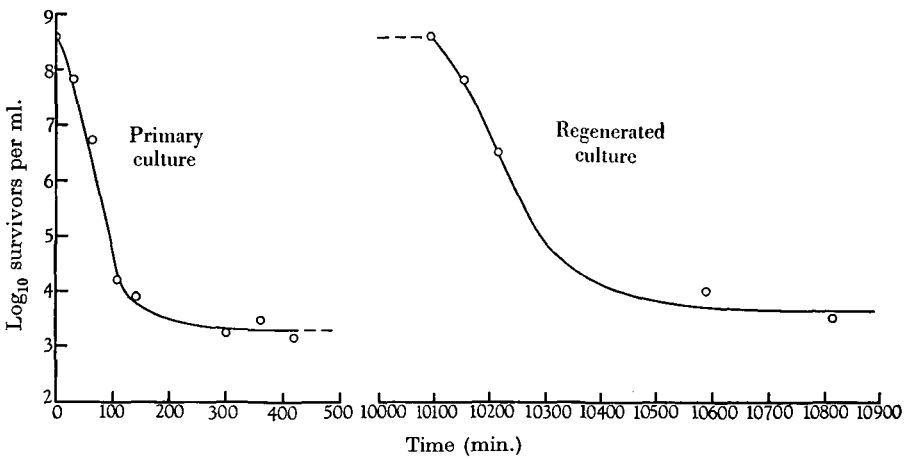

Fig. 2. Showing the relationship between logarithm of survivors and time for the primary and regenerated cultures of *Bact. coli* exposed to 53° C.

longer in the regenerated than in the primary cultures, the increase being 70 and 90 % at 49 and 53° C. respectively. The maximum death-rate fell, however, only by 25 % at the lower temperature and 50 % at the higher, and, since the maximum death-rate operates for only a part of each disinfection, this decrease is insufficient to account for the whole of the increase in the disinfection time. This fact is illustrated in Figs. 1 and 2, where it is not merely in an increased resistance of the hardier fraction. The breeding of a truly more resistant culture would thus seem to have been accomplished.

There can be no doubt of the real existence of the permanent population; the fact that viable cells persisted for long periods after the active disinfection was complete is clearly shown in Tables 1 and 2, where the times at which the phase of permanent population commenced are indicated. At 49° C.

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Table 1. *The relation between numbers of viable cells and time in a standard culture of* Bact. coli *subjected to alternate disinfection at* 49° C. *and regeneration at* 35° C.

First heat treatment at 49° C.			First regeneration at 35° C.		Second heat treatment at 49° C.			Second regeneration at 35° C.	
	Survivors per ml.					Survivors per ml.			
Time (min.)	No.	$\log_{10}$	Time (min.)	Survivors per ml.	Time (min.)	No.	$\log_{10}$	Time (min.)	Survivors per ml.
0	330,600,000	8·5193	3420	100,400	7,305	(268,100,000)	(8·4283)	18,945	42,680
60	296,200,000	8·4716	3635	35,940,000	7,450	196,600,000	8·2936	19,200	46,400,000
120	265,500,000	8·4240	4320	234,700,000	7,695	179,500,000	8·2553	19,425	854,500,000
240	205,300,000	8·3124	5070	242,800,000	7,995	114,700,000	8·0595	20,180	1,086,000,000
365	149,000,000	8·1732	5820	242,800,000	8,460	29,860,000	7·4751	20,475	1,136,000,000
485	117,100,000	8·0686	7240	268,100,000	8,745	15,940,000	7·2025	20,880	1,052,000,000
615	52,210,000	7·7178	7305*	—	9,000	9,327,000	6·9697	21,660	1,188,000,000
720	33,000,000	7·5185			9,375	2,910,000	6·4639	22,350	1,077,000,000
855	15,160,000	7·1807			10,185†	9,625	3·9834	23,055	1,153,000,000
1030	2,019,000	6·3051			10,605	479	2·6803	24,390	1,128,000,000
1140	1,404,000	6·1473			11,445	46	1·6628		
1260	658,000	5·8182			12,255	285	2·4548		
1380	348,000	5·5416			13,005	616	2·7896		
1565	98,490	4·9934			13,020‡	—	—		
1740	26,380	4·4213			13,095	3,353	3.5254		
1980†	6,164	3·7913			13,210	2,928	3·4666		
2280	17,070	4·2321			13,620	6,612	3·8203		
2910§	—	—			14,340	20,190	4·3051		
					16,035	9,620	3·9832		
					16,515	2,859	3·4562		
					17,595	22,460	4·3514		
					18,000	9,527	3·9789		
					18,705	9,532	3·9792		
					18,750§	—	—		

* Temperature raised to 49° C.
† Permanent population phase commenced.
‡ 2 g. Difco dehydrated broth added.
§ Temperature reduced to 35° C.

Table 2. *The relation between numbers of viable cells and time in a standard culture of* Bact. coli *subjected to alternate disinfection at* 53° C. *and regeneration at* 35° C.

First heat treatment at 53° C.			Regeneration at 35° C.		Second heat treatment at 53° C.		
	Survivors per ml.					Survivors per ml.	
Time (min.)	No.	$\log_{10}$	Time (min.)	Survivors per ml.	Time (min.)	No.	$\log_{10}$
0	386,700,000	8·5874	3,000	6,024	10,095	(387,900,000)	(8·5887)
30	64,870,000	7·8121	3,180	7,989	10,155	63,740,000	7·8044
60	5,098,000	6·7074	3,455	28,170,000	10,215	3,135,000	6·4962
105	14,710	4·1676	3,600	187,800,000	10,590*	9,827	3·9924
140*	7,293	3·8629	3,855	272,200,000	10,815	3,146	3·4977
300	1,647	3·2166	4,275	287,100,000	13,070	160	2·2041
360	2,814	3·4493	4,535	287,500,000	13,300	1,448	3·1608
420	1,361	3·1338	4,835	282,700,000			
510	1,619	3·2092	5,120	321,500,000			
695	732	2·8645	5,640	313,800,000			
780	6,492	3·8123	6,075	311,700,000			
870	2,162	3·3349	6,525	323,100,000			
1010	6,416	3·8073	7,440	348,300,000			
1110	217	2·3365	8,880	364,700,000			
1500	126	2·1004	10,065	387,900,000			
1740	704	2·8476	10,095†	—			
2880‡	—	—					

* Permanent population phase commenced.
† Temperature raised to 53° C.
‡ Temperature reduced to 35° C.

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(Table 1) this population was maintained for only about 1000 min. before regeneration was initiated, but at 53° C. (Table 2) this phase was prolonged for 2740 min., after the active disinfection had been accomplished in about 111 min. (Table 3). If regeneration had indeed resulted in a culture with enhanced resistance this effect should also have been reflected in an increased size of the permanent population after disinfection of the regenerated culture, but the data in Tables 1 and 2 do not reveal that any such increase had occurred, and in this respect therefore the regenerated cultures were not more heat resistant than their parents. However, the practical difficulty of obtaining reliable estimates of the numbers of viable cells in the presence of an overwhelming excess of dead cells may be responsible for obscuring any actual difference in viable cell level which may have existed.

had not been utilized. Lack of food was therefore unlikely to have been the cause of apparent fluctuations in the permanent population, especially since it is clear that following the 2 g. addition fluctuations still occurred though at a somewhat higher level than before.

As indicated above, another possibility is that the fluctuations originated from some technical difficulty in sampling, especially from the regenerated cultures, since through the successive disinfection and regeneration enormous numbers of dead cells must have accumulated, and they may have interfered with the obtaining of representative samples of viable cells when these were few in number. Direct microscopic counts were not made and the actual number of dead cells is not known. The turbidity of the cultures was measured frequently during these experiments in a Spekker photoelectric

Table 3. *The effect of regeneration on the heat resistance of cultures of* Bact. coli, *as shown by the changes in maximum death-rate and disinfection time*

Temperature of heat treatment (°C.)	Maximum death-rate (× 1000)		Disinfection time (min.)	
	Primary culture	Regenerated culture	Primary culture	Regenerated culture
49	2·981 ± 0·128	2·301 ± 0·358	1705 ± 25·3	2872 ± 144·3
53	38·670 ± 6·58	21·803*	110·9 ± 8·52	207·5*

* Two points only available for regression of log survivors on time.

During the permanent population phase the numbers of viable cells apparently fluctuated considerably, and it is conceivable that successive cycles of growth and death were occurring. At the elevated temperatures the metabolic rate of these cells might have been very high so that the growing cells quickly exhausted the available nutrients and then, in a starved condition, became more sensitive to heat so that death of many of the cells occurred. The decrease in number of viable cells would allow the food which was continually being added to accumulate and ultimately to lead to a fresh wave of growth. Considerations of this kind led to the decision to add 2 g. of dehydrated Difco broth to the culture at 49° C. at a time when the permanent population phase of the regenerated culture had become well established. The response to the additional food, however, was slight. It is true that the mean values of the permanent population before and after the addition of this food were 2210 and 9676 cells/ml. respectively, but in view of the marked fluctuations of the counts and their relative infrequency these values may not be significantly different. Moreover, when the temperature of this culture was reduced from 49 to 35° C. for the second time the response was striking, as a population density of over 1000 million viable cells per ml. was soon established. This points to the existence in the culture of a quantity of unused food and suggests that much of the 2 g. of additional food

absorptiometer, and by the use of an appropriate conversion factor these values could be converted into total cell numbers. A suitable conversion factor for the standard (unheated) culture has already been determined (Jordan & Jacobs, 1944*b*), but the changes in total cell numbers during these disinfections could not be followed since there is evidence that the factor changed during the heat treatment. As soon as the lethal temperature was attained the turbidity immediately began to fall, sharply at first and later tending to reach a constant or slowly diminishing value. This decrease was attributed to shrinkage of the cells when exposed to heat and was confirmed by microscopical examination. Some graphs illustrating this change in turbidity are given in Fig. 3, the decrease during heat treatment being plainly shown. During the permanent population phase the turbidity either remained constant or decreased slowly. If growth of the surviving cells did occur at the elevated temperature the additional turbidity thereby induced was evidently insufficient to offset the tendency to decline and might in any case have been too small to detect. The turbidity measurements therefore do not help to answer the question whether cell multiplication occurred during the stable population phase. The viable cell values recorded in Tables 1 and 2 were all obtained from colony counts on plates incubated at 35° C., and it by no means follows that cells capable of growth and division on the solid medium

at 35° C. were similarly active in the liquid culture at 49 or 53° C. In the experiment at 49° C. duplicate sets of plates were made from samples taken during the permanent population phase, one set being incubated at 35° and the other at 49° C. While the former soon showed colonies the latter remained clear, and evidently growth on agar at 49° C. was either not possible or extremely slow. However, living cells persisted on the latter plates throughout the incubation at 49° C., since on transference of the plates to 35° C. visible colonies were formed within a further 48 hr. Again, in the experiment at 53° C. tubes of nutrient broth were inoculated from the culture during the phase of permanent population, some being incubated at 35° and the remainder at 53° C. The former soon showed abundant growth, but the latter remained clear. The evidence thus suggests that during the permanent population phase the viable cells were not multiplying in the culture or doing so only very slowly. The observed fluctuations in the population are therefore not definitely attributable to alternate waves of growth and death, and some technical difficulty, the nature of which has yet to be elucidated, was probably responsible.

In order to obtain more detailed information about the change in heat resistance induced by regenerating fresh cultures from the survivors of a disinfection, it was decided to carry out a series of heat treatments and regenerations on the same culture. A temperature of 51° C. was employed, and altogether the culture was regenerated and disinfected four times after the heat treatment of the primary culture. The experiment occupied 20 days, and during this time the accumulated products of metabolism led to a change in reaction of the culture from *p*H 7·0 to 7·3. The detailed results are given in Table 4. Each period of heat treatment lasted approximately the same time (2855–2985 min.), except the last, which was terminated after 1680 min. The periods of regeneration lasted for an equal time (2805–2925 min.), except the final one, which was continued for approximately twice as long. These times are important because, other things being equal, the regenerated cultures, except the last, ought to have reached the same number of viable cells in the equal periods allowed. But, as shown in Table 5, whereas the number after the first regeneration was lower than that of the primary culture, it rose in subsequent regenerations until after the third there was a larger population than in the primary culture. In the fourth regeneration the longer time allowed led to considerably enhanced numbers but even halfway through this regeneration (after only 2180 min.) the viable population, at approximately 470 million cells per ml., was higher than that of the primary culture. This suggests that food had gradually accumulated during the experiment through failure of the cells to utilize completely those portions which were added during the periods of heat treatment.

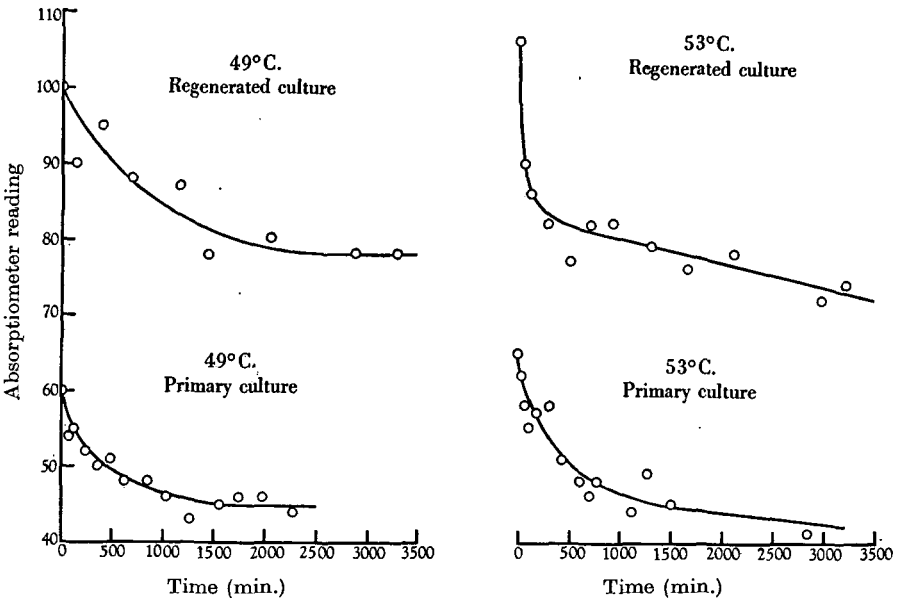

Fig. 3. Showing the relationship between absorptiometer reading and time for the primary and regenerated cultures of *Bact. coli* exposed to 49° and 53° C.

Table 4. *The relation between numbers of viable cells and time in a standard culture of* Bact. coli *subjected to alternate disinfection at* 51° C. *and regeneration at* 35° C.

First heat treatment at 51° C.			First regeneration at 35° C.		Second heat treatment at 51° C.			Second regeneration at 35° C.	
	Survivors per ml.					Survivors per ml.			
Time (min.)	No.	$\log_{10}$	Time (min.)	Survivors per ml.	Time (min.)	No.	$\log_{10}$	Time (min.)	Survivors per ml.
0	355,900,000	8·5513	4,320	230,700,000	5,745	(270,300,000)	(8·4319)	8,810	4,708
60	112,800,000	8·0520	5,000	240,000,000	5,805	165,100,000	8·2178	8,960	179,600
130	25,040,000	7·3986	5,705	270,300,000	5,865	86,830,000	7·9387	9,185	81,170,000
180	6,090,000	6·7846	5,745*	—	5,960	36,980,000	7·5680	9,410	271,400,000
310	163,200	5·2127			6,055	10,480,000	7·0203	9,905	278,700,000
410	28,850	4·4602			6,355	6,977	3·8436	10,145	273,400,000
600†	844	2·9263			6,535†	1,493	3·1741	10,835	303,600,000
780	1,083	3·0346			6,800	513	2·7101	11,480	319,000,000
960	10,100	4·0043			6,945	2,212	3·3488	11,525*	—
1,150	196	2·2923			7,100	1,505	3·1775		
1,320	228	2·3579			7,355	22,660	4·3553		
1,570	1,425	3·1538			7,695	410	2·6128		
1,930	479	2·6803			8,540	4,490	3·6522		
2,860‡	—	—			8,600‡	—	—		

Third heat treatment at 51° C.			Third regeneration at 35° C.		Fourth heat treatment at 51° C.			Fourth regeneration at 35° C.	
	Survivors per ml.					Survivors per ml.			
Time (min.)	No.	$\log_{10}$	Time (min.)	Survivors per ml.	Time (min.)	No.	$\log_{10}$	Time (min.)	Survivors per ml.
11,525	(319,000,000)	(8·5038)	14,675	923	17,315	(414,700,000)	(8·6177)	20,840	129,200,000
11,585	143,600,000	8·1571	15,050	3,077,000	17,375	124,000,000	8·0934	21,665	392,100,000
11,660	69,160,000	7·8399	15,830	374,000,000	17,435	64,610,000	7·8103	23,060	469,800,000
11,735	38,920,000	7·5901	16,535	416,000,000	17,495	31,620,000	7·5000	25,895	735,300,000
11,825	12,370,000	7·0923	17,270	414,700,000	17,585	5,346,000	6·7280	25,940*	—
11,930	3,365,000	6·5269	17,315*	—	17,675	833,400	5·9208		
12,125	62,380	4·7951			17,825	39,870	4·6007		
12,305†	2,918	3·4651			17,915†	3,796	3·5793		
12,515	5,084	3·7062			18,440	502	2·7007		
12,800	6,874	3·8373			18,485	217	2·3365		
13,370	889	2·9489			19,100	7,672	3·8849		
13,655	5,700	3·7559			20,270‡	—	—		
14,510‡	—	—							

Fifth heat treatment at 51° C.		
	Survivors per ml.	
Time (min.)	No.	$\log_{10}$
25,940	(735,300,000)	(8·8665)
25,970	312,600,000	8·4950
26,030	94,500,000	7·9754
26,090	33,920,000	7·5305
26,210	2,808,000	6·4484
26,270	815,400	5·9114
26,320	98,960	4·9955
26,465†	923	2·9652
26,585	684	2·8351
26,645	3,181	3·5025
27,350	296	2·4713
27,620	1,482	3·1709

* Temperature raised to 51° C.
† Permanent population phase commenced.
‡ Temperature reduced to 35° C.

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Two aspects of the effect of successive regeneration on the heat resistance of the culture can be seen from Table 5. The maximum death-rates are there given for successive disinfections, calculated as before on the assumption that the rate was constant between the survivor levels of 10 and 0·01 %, together with the corresponding disinfection times, which are counted from the start of each period of heat treatment. In successive regenerations the maximum death-rate decreased and then increased again, the minimum value being in the primary culture, yet though the maximum death-rate was the same, the disinfection time was only slightly enhanced. Evidently other factors were operating, and the resistance of the regenerated cultures was altering in a complicated manner. Some indication of these effects can be seen from Fig. 4, in which the logarithms of survivors are shown plotted against time for the successive disinfections. The once- and twice-regenerated cultures behaved somewhat similarly to the regenerated cultures at 49 and 53° C., in that the

Table 5. *The change in heat resistance, as shown by the maximum death-rate and disinfection time, in cultures of* Bact. coli *subjected to alternate disinfection at* 51° C. *and regeneration at* 35° C.

Type of culture	Initial population (millions/ml.)	Maximum death-rate (× 1000)	Disinfection time (min.)
Primary	355·9 ± 12·38	10·674 ± 0·770	389·9 ± 12·41
First regeneration	270·3 ± 12·38	9·709 ± 0·934	560·2 ± 23·45
Second regeneration	319·0 ± 4·52	7·200 ± 0·669	656·2 ± 29·07
Third regeneration	414·7 ± 13·35	8·804 ± 0·044	508·3 ± 1·07
Fourth regeneration	735·3 ± 28·47	10·536 ± 1·252	411·1 ± 18·37

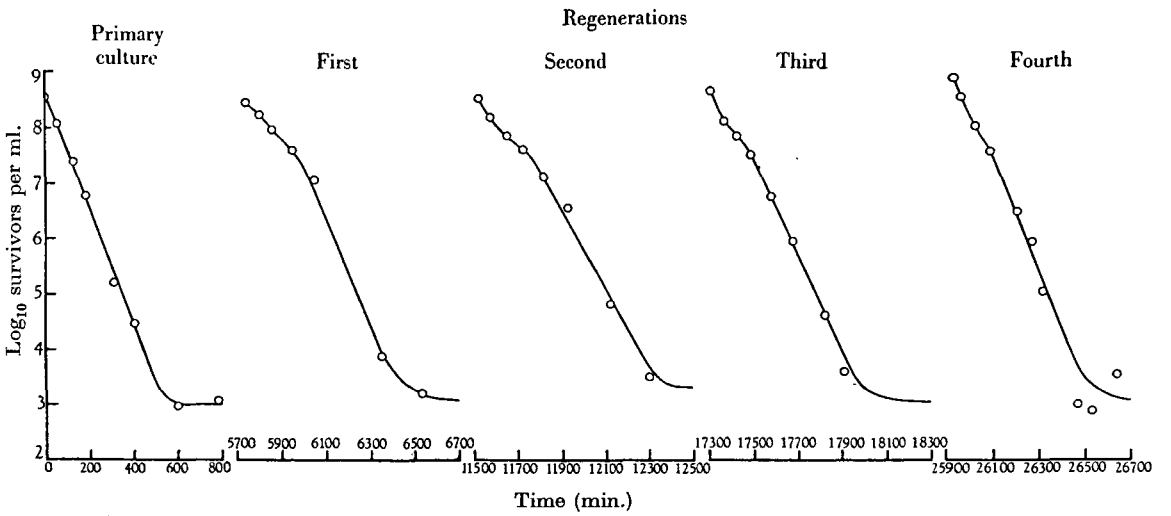

Fig. 4. Showing the relationship between logarithm of survivors and time for the primary and successively regenerated cultures of *Bact. coli* exposed to 51° C.

obtained after the second regeneration. Correspondingly, the disinfection time reached a maximum after the second regeneration. It cannot be said, however, that the change in maximum death-rate was the sole factor controlling the disinfection time, since the initial populations were not equal. The once-regenerated culture had a smaller initial population than the primary, but while the maximum death-rate was only about 10 % lower, the disinfection time was increased by nearly 50 %. Again, after the fourth regeneration the initial population was more than double that resistance of the cultures as a whole increased. The influence of the second regeneration was comparatively slight, but, in fact, the upper part of its graph dips slightly below that for the once-regenerated culture, in spite of having started from an initially higher value. With the third and fourth regenerations there is distinct evidence of an accelerated death-rate in the early stages, and it is clear that had the curve for the fourth regeneration commenced at the same point as that for the primary culture, the disinfection would have been accomplished in a shorter time. The disinfection

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time shown in Table 5 for the four times regenerated culture is thus artificially high and the impression of slightly enhanced resistance is illusory.

To determine the changes in the death-rate in the early parts of the successive disinfections each set of data was plotted on a large scale, and from the smooth curves drawn through these points values of log survivors at short equal intervals of time were read off. From these readings the death-rates were calculated, and as shown in Fig. 5 the death-rate of the primary culture was low at first but rose steadily as the reaction proceeded until the constant maximum was reached. The once-regenerated culture behaved similarly except that the initial death-rate was lower than before and remained constant for a considerable time. The subsequent increase was faster but the maximum rate not so high. The twice-regenerated culture behaved differently in that the death-rate, which was initially almost as high as that in the primary culture, decreased sharply for a time but subsequently rose equally rapidly to a maximum which was, however, below that of the once-regenerated culture. After three regenerations the initial death-rate was clearly faster than that in the primary culture, but there was a very sharp fall to a minimum value which was somewhat higher than that for the twice-regenerated culture. The increase after the minimum was rapid, but the maximum attained was still below that of the primary culture. After the fourth regeneration the initial death-rate was but slightly further increased, the subsequent fall less sharp and the minimum not so low. The succeeding increase was rapid and the maximum attained approximately equal to that in the primary culture. These regenerated cultures therefore showed systematic departures from the log survivors-time pattern common to the primary cultures as established by Jordan *et al.* (1947), and the nature and extent of the departure varied with the treatment that the culture had received. Fig. 6 shows how the death-rate varied in the experiment at 49° C. The primary culture exhibited a brief period of low constant death-rate, and the graph is intermediate in type between those for the primary and once-regenerated cultures at 51° C. The regenerated culture at 49° C. behaved more like the thrice-regenerated culture at 51° C. Observations in the experiment at 53° C. were not sufficiently numerous for the construction of reliable graphs showing changes in death-rate. In an analysis of this kind variations are to be expected, and may be ascribed to the difficulty of drawing the best smooth curves through a series of points, especially when the positions of these points are, by virtue of the laws of random sampling, subject to some degree of inherent uncertainty. Nevertheless, it seems evident that there are regular trends in the changes of death-rate with time in these regenerated cultures due to the successive heat treatments.

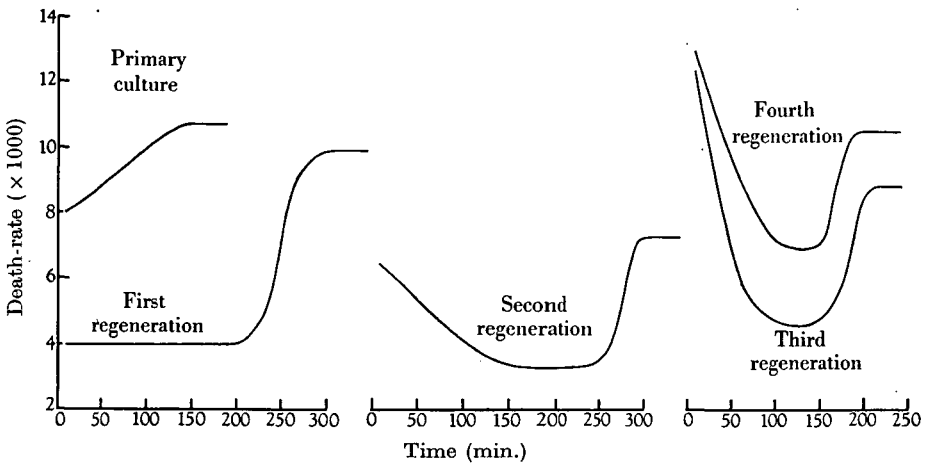

Fig. 5. Showing the relationship between death-rate and time for the primary and successively regenerated cultures of *Bact. coli* exposed to 51° C.

In the experiment at 51° C. the phases of permanent population which existed between the active disinfections and regenerations were in all four cases of approximately equal length, ranging from 2065 to 2355 min. as appears from Table 4, where the times at which these phases began and ended are indicated. During these periods the counts fluctuated widely as in the previous experiments. Often the counts indicated only a few hundred viable cells per ml., but at least as frequently the numbers were in the thousands. Reference has

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been made above to the possibility that the accumulation of dead cells, which must have numbered several million times as many as the living cells, may have rendered it difficult to obtain representative samples of the latter from the culture for estimation. If this were so, the fluctuations during the fourth and fifth periods of permanent surviving

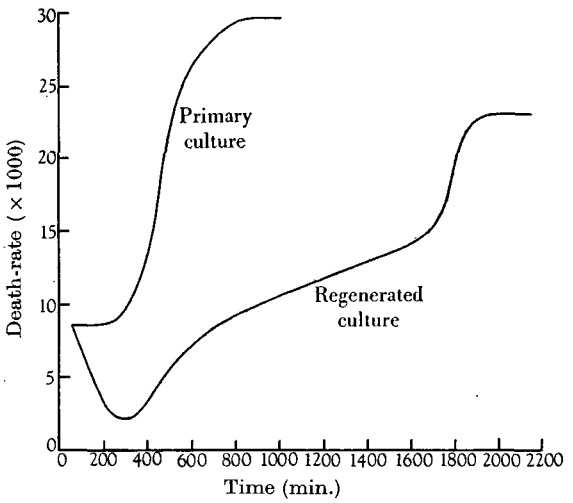

Fig. 6. Showing the relationship between death-rate and time for the primary and regenerated cultures at 49° C.

population should have been more prominent than in the first period. Table 4 shows that there is no evidence of this, the fluctuations during the first period being quite as marked as in subsequent periods. In these circumstances, mean values for the surviving populations may not have any real significance, yet it is interesting to note that these means, which have values of 2051, 4755, 4293, 3047 and 1313 cells/ml. for the successive periods, do not show any consistent tendency to increase. Indeed, bearing in mind the very high viable population at the start of the fifth disinfection, the relative level of the resulting surviving population is below that which remained after the primary disinfection. There is thus no evidence that by repeated regeneration from the surviving cells of a heat-treated culture the percentage of resistant cells can be increased.

DISCUSSION

The experimental work reported above has shown that in *Bact. coli* cultures subjected to heat a small proportion of the cells is very resistant and able to withstand exposure for long periods, if not indefinitely, to temperatures which are rapidly fatal to the vast majority. This phenomenon, it must be stressed, is quite different from the prolonged survival of a few cells in a culture where progressive diminution in viable count is occurring. That situation reflects the fact that bacteria exposed to lethal agencies diminish in viable numbers according to a logarithmic law, although the logarithmic death-rate need not be constant throughout a disinfection. Beamer & Tanner (1939) showed that in suspensions of *Salmonella* species exposed to temperatures between 55 and 60° C. there was a diminution in the death-rate above 99·99 % mortality, although it is clear that the cells continued to die but at a slower rate. These authors' experiments were of short duration, and it is possible that had they been extended the existence of a few cells capable of indefinite survival would have been detected.

In the present work the change to the condition of permanent surviving population showed itself in a sudden cessation of mortality, and subsequently there may even have been an increase in numbers of viable cells, but the evidence for this is not conclusive. The plate counts at 35° C. suggested fluctuating numbers, but the apparent failure to grow of subcultures on agar plates incubated at 49° C. or in tubes of broth at 53° C. tends to show that no multiplication was occurring in the parent culture. This lack of growth on subculture may, however, have been misleading, as Nahgski, White & Hoover (1944) found that large numbers of mesophilic bacteria were present in fermentations carried out at 52° C. These cells grew on agar plates at 37° C. but refused to develop under otherwise similar conditions at 52° C. These authors considered that the mesophilic organisms were in fact active at the higher temperature in the fermenting material, and it seems that there may be some difficulty in obtaining growth on solid media, or even in different liquid media, of mesophilic organisms which have previously been induced to function thermophilically. The permanent survivors of the present experiments may perhaps be regarded as constituting thermophilic cultures with the fluctuations in numbers representing short growth phases followed by rapid phases of decline. Imšenecki & Solnzeva (1945) found the generation time of thermophilic organisms to be short compared with that of mesophiles, and that in broth the phase of decline set in early and was very rapid. If the surviving cells were really behaving as a thermophilic culture, then adaptation must have occurred. Bacteria are well known for their powers of adaptation to changed environments, and the acquisition of characters such as drug-fastness and the ability to use different substrates is well established. The adaptation may occur either through the alteration of the characters of certain cells (Lodge & Hinshelwood, 1944) or by the selection of naturally resistant strains (Severens & Tanner, 1945). Adaptation to heat has not frequently been reported. Perhaps the clearest

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example in the literature is the demonstration by Baars, quoted by Bunker (1936), of the conversion of the mesophilic *Vibrio desulphuricans* to the thermophilic habit, but even here the adaptation could only be accomplished by gradual acclimatization. Shock tactics such as were used in the present investigation proved useless. Also, the transformation was reversible, and in the present case prompt reversion may have occurred when the temperature was reduced to 35° C., since rapid growth soon resulted.

With the evidence at present available the permanent survivors could equally well be regarded as inactive in the sense that multiplication was not occurring. Whether they had acquired the ability to withstand high temperatures by a process of adaptation, or whether they were the most resistant cells from a population possessing a wide range of susceptibility to heat, it might have been anticipated that the numbers of these cells would be smaller at higher temperatures, but there was no evidence of this. The fluctuation in numbers, the reason for which is not known, makes it difficult to assess the true position, but Nelson (1943, 1944) has shown that cells which have survived heat treatment are more exacting in their nutritional requirements for growth. Small variations in cultural conditions may therefore have greatly affected the apparent numbers of survivors, and this possibility raises the whole question of when a bacterial cell should be considered dead.

Variation in resistance between the individual cells of a culture is known to occur as shown by Yesair & Cameron (1936), who reported that the spores of several species of bacteria could be separated by centrifugation methods into fractions having different heat resistance. Also, it is well known that cells in the logarithmic phase of growth are much less resistant than those in the stationary phase, and the standard cultures used in the present work probably contained both actively dividing and non-proliferating cells.

Whether the character of resistance had been acquired or was inherent in certain cells, their descendants, although formed at a lower temperature, might show an enhanced heat resistance. This might be manifest in an increased ability to adapt, or result in a culture whose constituent cells had generally a greater resistance. The fact that greater resistance was shown by the once- and twice-regenerated cultures points to the conclusion that the character had in fact been transmitted. In contrast, however, Gage & Stoughton (1906) failed to show that the very marked heat resistance of certain cells in *Bact. coli* cultures was inheritable. Again, attempts by various workers to enhance the resistance of bacterial spores by culturing from the more resistant individuals have given variable results, except perhaps in the case of *B. mycoides* (Magoon, 1926; Williams, 1936), and it is clear that many factors are involved and conclusive results difficult to obtain. In the present work the resistance apparently reached its peak after two regenerations, and this suggests that extraneous factors were at work, some operating to produce a simulation of increased resistance and others having the reverse effect. The conditions in which cells are heated greatly affect their survival, and during the successive heatings and regenerations some environmental changes must have occurred. For example, dead cells were accumulating, and these may have exerted an influence, as Lange (1922) showed that the presence of heat-killed cells from young cultures decreased the death-rate of *Bact. coli*. Further, there was most probably an accumulation of organic matter which may have had a protective influence. But both these changes were progressive and should have led to a continuously increasing 'resistance' so that other factors tending to raise the death-rate must have become increasingly important after the second regeneration. One of these may have been the accumulation of metabolic products of an alkaline nature, since at the end of the very long experiment at 51° C. the *p*H of the culture had risen from 7·0 to 7·3, and alkalies are known to be potent germicides, particularly at high temperatures.

It has also been shown above that the successive regenerated cultures differed from one another in the general pattern of their heat resistance, especially in the early stages of the disinfection. The initial death-rate was lower in the once-regenerated culture than in the primary, but in the following regenerations it rose very markedly. Among factors which could have caused such an increase is the composition of the culture in respect of cells of different ages. Young cells die more rapidly than older ones, and it is possible that the proportion of young cells was increasing and thereby obscuring the evidence of an enhanced resistance in the culture as a whole. After the fourth regeneration the further increase in the initial death-rate was slight and the postulated change in age distribution may have reached its limit. If the cultures had become steadily richer in young cells during successive regenerations, the protective effect of the dead cells should have become increasingly prominent, as it is only killed young cells that act in this manner. Relevant also to this question of the magnitude of the initial death-rate is the fact shown by Watkins & Winslow (1932) that the rate of death of washed bacteria exposed to heat depends on the initial concentration of organisms. As the concentration is increased the rate is decreased. Since after the fourth regeneration the culture contained nearly twice as many

viable cells as after the third, this may have induced a reduction in the initial death-rate sufficient to offset a tendency otherwise to increase. It is noteworthy that the once-regenerated culture contained fewer viable cells than the primary and should on this account have shown a faster death-rate. The fact that the death-rate was actually slower is additional evidence in favour of the hypothesis that a true increase in resistance had occurred in this culture.

Evidently, by the technique of successive regeneration of fresh cultures in the same flask a conclusive answer cannot be given to the question whether the high resistance to heat shown by the permanent survivors can be transmitted and a culture with greatly enhanced resistance built up. Too many factors with opposed tendencies become operative, and the extent of their action and the time when this becomes appreciable are not known. It will be necessary to conduct experiments in which the surviving cells from one disinfection form the inoculum for a fresh culture in a different flask but under otherwise similar conditions and to examine the heat resistance of a series of such cultures. By this means many, if not all, of the disturbing factors will be eliminated and a definite solution to the problem obtained.

SUMMARY

1. An attempt has been made to discover the origin and nature of the small proportion of the cells in standard cultures of *Bact. coli* which is able to withstand, apparently indefinitely, exposure to temperatures of 49, 51 and 53° C.

2. The method adopted was to allow these survivors to generate fresh cultures in the same flask merely by reducing the temperature to 35° C. and to subject the resulting cultures in turn to heat treatment. Only one process of regeneration and subsequent disinfection was carried out at 49 and 53° C., but at 51° C. the process was repeated four times. The disinfection curve was determined on each occasion and the permanent population allowed to become established.

3. Changes in the heat resistance of the cultures were judged by (*a*) the disinfection times (99·99 % mortality), (*b*) the maximum death-rates, (*c*) the levels of the surviving populations and (*d*) the variations in death-rate with time during each disinfection.

4. Marked fluctuation in numbers of viable cells was observed during each of the phases of permanent surviving population. The exact levels of survivors were therefore uncertain, but after exposure to 49° C. the mean values were not higher than when 53° C. was used, nor was there an increase in the mean numbers of survivors in the successively regenerated cultures.

5. The survivors did not grow when inoculated into either nutrient agar plates or tubes of broth held at the respective high temperatures, although they remained alive and developed readily on being transferred to 35° C.

6. The maximum death-rate decreased and the disinfection time increased markedly after one regeneration of the cultures. The increase in the latter was not wholly due to the decrease in the former, as the death-rates throughout the disinfection of the regenerated culture were lower.

7. After the second regeneration (at 51° C.) the maximum death-rate declined and the disinfection time rose still higher, but the process was reversed after the third and fourth regenerations.

8. The shape of the graph of log survivors against time altered markedly after the second regeneration at 51° C. The initial death-rate was higher but fell sharply to a minimum before rising again to the final maximum.

9. The experimental findings are discussed. An increase in the general heat resistance of these cultures may have occurred as a result of one regeneration, but the technique employed permitted the gradual alteration of the environmental conditions, and this appears to have exerted complex effects on the cultures so that the results of further regenerations are inconclusive.

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