STUDIES ON BACTERIAL POPULATIONS IN SOLUTIONS OF PHENOLS

PART I. THE VIABILITY OF *Bacterium coli* IN AQUEOUS SOLUTIONS OF BENZYLCHLOROPHENOL

BY H. S. BEAN and V. WALTERS

From the Pharmaceutics Laboratories, School of Pharmacy, Chelsea Polytechnic, London, S.W.3

Received July 4, 1955

ANALYSES of the mortality of bacteria in aqueous solutions of bactericides have produced two major theories about the nature of the bactericidal reaction. The earlier theory, which has been referred to as the "mechanistic theory" likened the bactericidal reaction to a chemical reaction. Madsen and Nyman[1] and Chick[2] postulated that the mortality rate of organisms in a bactericidal system approximated closely to that which would be expected if the bactericidal reaction was a monomolecular one. This would imply a constant death-rate throughout the entire course of the reaction. The experiments of Chick show, however, that there is often a marked variation of the death rate during the reaction. Further, the majority of a large number of experiments described in the literature and analysed by Rahn[3] show a departure from the monomolecular rate. The alternative theory—the "vitalistic theory"—is based on the distribution of resistances of the individual organisms. The theory was supported by Hewlett[4], Reichel[5], Reichenbach[6] and by Henderson Smith[7,8]. Withell[9] demonstrated the existence of a rectilinear relation between probit-mortality and a simple transformation of contact time, such as the logarithm of the contact time. Neither Berry and Michaels[10,11], nor Jordan and Jacobs[12] accepted unconditionally the relation suggested by Withell, and have discussed the significance of the shape of the probit-mortality log-time curve.

The mechanistic and vitalistic theories offer different explanations for the course of the bactericidal reaction, but they have in common the axiomatic concept that the longer the time of contact between bacteria and bactericide the smaller the number of living organisms in the system. It is partly the intention of the present communication to demonstrate that, under appropriate conditions, there may be marked departures from the anticipated course of the bactericidal reaction and therefore, in the number of living organisms in the system.

It has been shown by several workers[13,14,15,16] that when bacteria are treated with certain bactericides, there is a leakage into the surrounding medium of nitrogenous materials, such as purines and pyrimidines which are characterised by having a maximal ultra-violet absorption at a wavelength of 260 mμ. Salton and Alexander[13] and Salton[14] have indicated that there is a relation between the amount of cetrimide added to a suspension of *Staphylococcus aureus* and the amount of 260 mμ absorbing material released from the cells, the latter also being increased by an

elevation of temperature. Newton[15] reported that the antibiotic polymyxin E produced a release of 260 mμ absorbing material from washed cells of *Pseudomonas aeruginosa*. Few and Schulman[16] found a release of similar material from a number of species of bacteria when they were treated with polymyxin E. The results of the experiments published by Salton[14], Few and Schulman[16] and by Newton[15] indicate that an increase in the severity of the bactericidal conditions leads to an increase in the amount of 260 mμ absorbing material released from the cells. The present communication examines whether a correlation exists between the mortality of certain bacteria in an aqueous system and the amount of 260 mμ-absorbing material released from the cells.

Materials and Methods

The Organism

Bacterium coli (N.C.T.C. No. 5933) was selected for the present study since it had been used satisfactorily in earlier disinfection studies by Berry and Michaels[17] and Berry and Bean[18]. Further, the latter authors used it in conjunction with the same bactericide that was selected for the present experiments. The organism was maintained by freeze-drying from nutrient broth.

A tube of freeze-dried material was reconstitued at monthly intervals by suspension in horse meat infusion broth and the suspension used in a manner similar to that described in the British Standard Specification for the Rideal Walker Test (B.S.S. No. 541: 1934—amended May 1951). Three stock agar slope cultures were prepared from the reconstitued material, 2 of them were reserved and the third used for the preparation of daily slopes during the first part of a month. Subcultures were made from the stock slope into broth at 24-hour intervals for 3 days, 2 ml. of the third subculture being distributed over the surface of an agar slope and incubated at 37° C. for 16 hours. This procedure was repeated until the fourteenth subculture, when the cycle was recommenced from one of the reserved stock slopes.

Preparation of the Bacterial Suspensions

The 16-hour growth was washed from the slope with quarter-strength Ringer's solution and the resulting suspension centrifuged at about 1000 r.p.m. to remove any small lumps of agar removed with the organisms. The supernatant was removed and the organisms washed 3 times in quarter-strength Ringer's solution using about 100 ml. solution in all. After the third washing the supernatant was removed, and the closely packed wet cells resuspended to produce a concentration of approximately 2000×10^6 per ml. in the solution under test. The cell concentration was adjusted with the aid of an Eel nephelometer previously standardised against suspensions of the same organism which were counted using darkground illumination in a Thoma hæmocytometer cell (depth 1/50 mm.).

The Bactericide

The bactericide used in the experiments was benzylchlorophenol (5-chloro-2-hydroxy diphenylmethane). It had a m.pt. of 48·5° C. and

its molecular extinction $\epsilon_{max.}$ at $\lambda_{max.}$ 282 mμ in water was found to be 2310. The water solubility was 1 in 6380, determined by means of a Unicam S.P. 500 spectrophotometer. (Allawala and Riegelman[19] give $\epsilon_{max.}$ 2360 at λ 282 mμ in water and a water solubility of 1 in 6300). Figure 1 shows the compliance of aqueous solutions of benzylchlorophenol with the Beer-Lambert law, and provides a reference graph from which the concentrations of unknown solutions can be read.

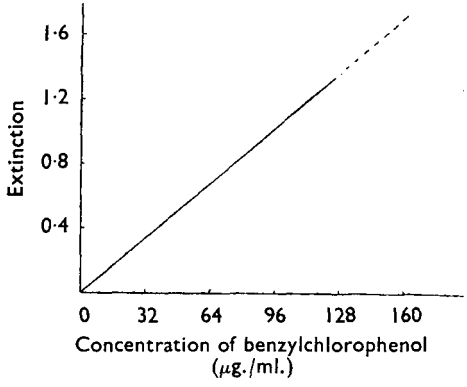

FIG. 1. Relationship of the extinction at 282 mμ to benzylchlorophenol concentration.

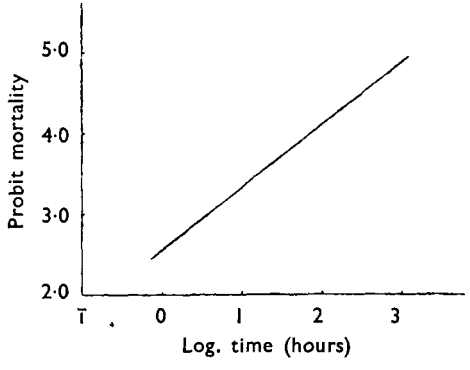

FIG. 2. Relationship of probit mortality to log contact time of *Bact. coli* with distilled water.

The Bacteriological Method

The roll-tube capillary dropping pipette technique described by Wilson[20], Withell[21] and by Berry and Michaels[17] was used for all viable counts. The method is economical both of materials and space and, with experience, it yields accurate results[17,22].

For each viable count five roll-tubes were prepared and the mean count calculated.

Estimation of the soluble cell constituents or Benzylchlorophenol in the supernatants from bacterial suspensions

8 to 10 ml. samples of the cell suspension were taken at the time of making viable counts and the majority of the cells removed by centrifugation at x 8500 *g* for 3 minutes. The supernatant was then subjected to a further centrifugation at x 8500 *g* for 3 minutes. The ultra-violet absorption spectra of the cell-free supernatant fluids were examined using a Unicam S.P. 500 spectrophotometer. Where necessary corrections were made for the absorption of the suspending fluids.

ESTIMATES OF EXPERIMENTAL ERRORS

The sources of error in viable count techniques have been discussed by Berry and Michaels[17] and by Bullock, Keepe and Rawlins[22]. The major errors may be assumed to be due to variations in the capillary pipettes, variations in the volume of bacterial suspension measured by the pipettes, the non-homogeneity of the suspension, batch to batch variation in the

nutrient medium, and to personal errors in counting the colonies in the roll tubes after incubation.

Personal Error due to Counting

The causes of errors in counting bacterial colonies have been discussed by Wilson[23]. The magnitude of the error was estimated by the same author and by Berry and Michaels[17] and by Bullock, Keepe and Rawlins[22]. Table I shows the results during the present experiments when the colonies in each of 10 roll tubes were counted on each of 3 different occasions.

The standard error of the mean of 3 counts is 1·90, compared with the value of 1·65 obtained by Bullock, Keepe and Rawlins[22] and of 2·41 obtained by Berry and Michaels[17]. This value may be regarded as satisfactory.

TABLE I

ESTIMATION OF THE PERSONAL ERROR DUE TO COUNTING COLONIES IN 10 ROLL TUBES ON EACH OF THREE OCCASIONS

Roll tube No.	Counts (x) $n = 3$	Total of three counts $\Sigma(x)$	Mean $\bar{x}$	Corrected sum of squares (SS) $\Sigma(x-\bar{x})^2 = \Sigma(x^2) - \frac{\Sigma^2 x}{n}$			Variance = SS/N where N = 2
				$\Sigma(x^2)$	$\Sigma^2(x)/n$	SS	
1	137,135,141	413	137·7	56875	56856	19	9·5
2	146,150,147	443	147·7	65425	65416	9	4·5
3	252,247,246	745	248·3	185029	185008	21	10·5
4	195,190,188	573	191	109469	109443	26	13
5	237,230,231	698	232·7	162430	162401	29	14·5
6	182,188,190	560	186·6	104568	104533	35	17·5
7	99,102,100	301	100·3	30205	30200	5	2·5
8	127,129,133	389	129·6	50459	50440	19	9·5
9	135,129,132	396	132	52290	52272	18	9
10	131,129,137	397	132·6	52571	52536	35	17·5
	Totals	4915				216	

Total sum of squares for 30 observations = 216. Sum of squares for count variation $\frac{\Sigma SS}{\Sigma N} = \frac{216}{20} = 10{\cdot}8$.

Standard deviation of count variation = $\sqrt{10{\cdot}8} = \underline{3{\cdot}286}$. Standard error of mean of 3 counts = $\sqrt{\frac{10{\cdot}8}{3}}$ = $\underline{1{\cdot}897}$.

Errors due to Batch to Batch Variation in the Agar Medium

The influence of batch to batch variation in the agar has been examined by other workers[17,22], who have concluded that, provided the medium is prepared in a satisfactory manner, batches of reproducible sensitivity are obtained. The problem was not reinvestigated during the present experiments.

Suitability of the Viable Count Technique

In accordance with the recommendations of Thornton[24], the suitability of the nutrient medium employed for a viable count is usually expressed by calculating the Index of Dispersion for the colonies in a number of roll tubes. Table II shows the Index of Dispersion for 20 roll tubes for a viable count on a suspension of *Bact. coli* in quarter strength Ringer's solution. Reference to tables of χ^2 indicates a probability of 0·9 to 0·8, and thus the medium may be regarded as satisfactory.

The Index of Dispersion examines rather more than the suitability of the medium and includes the whole of the technique involved in making

20 replicate counts on a suspension of organisms. Thus the high value of P obtained is a favourable comment on the technique as a whole.

The overall errors involved in performing replicate counts on a series of bacterial suspensions are tested by a rather more comprehensive test, the basis of which is described by Berry and Michaels[17]. Table III records the Goodness of Fit of χ^2 values for 50 sets of five replicate roll tubes for counts of suspensions of *Bact. coli* which have not been in contact with a bactericide.

TABLE II

GOODNESS OF FIT OF χ^2 FOR 20 ROLL-TUBES USED IN ESTIMATING THE SUITABILITY OF A BATCH OF NUTRIENT AGAR

Counts (x)	Mean count ($\bar{x}$)	Deviations from mean ($x - \bar{x}$)	$(x - \bar{x})^2$
100		−14·75	217·56
99		−15·75	248·06
126		11·25	126·56
117		2·25	5·06
109		−5·75	33·06
120		5·25	27·56
123		8·25	68·06
118		3·25	10·56
117	114·75	2·25	5·06
106		−8·75	76·56
125		10·25	105·06
121		6·25	39·06
118		3·25	10·56
114		−0·75	0·56
122		7·25	52·56
121		6·25	39·06
105		−9·75	95·06
120		5·25	27·56
100		−14·75	217·56
114		−0·75	0·56
Σx = 2295		$\Sigma(x - \bar{x})$ = 0·00	$\Sigma(x - \bar{x})^2$ = 1405·7

$$= \frac{\Sigma(x - \bar{x})^2}{\bar{x}} = \frac{1405{\cdot}7}{114{\cdot}75} = 12{\cdot}25;\ N = 19;\ P = 0{\cdot}9\text{–}0{\cdot}8$$

TABLE III

GOODNESS OF FIT OF THE DISTRIBUTION OF χ^2 VALUES FOR VIABLE COUNTS ON 50 SETS OF FIVE REPLICATE TUBES, USING *Bact. coli* SUSPENDED IN DISTILLED WATER OR PHOSPHATE BUFFER

Value of χ^2	Expected frequency (m)	Observed frequency ($m + x$)	Difference (x)	x^2	$\frac{x^2}{m}$
Under 1·00	4·51	6	+1·49	2·25	0·50
1·00–1·99	8·70	15	+6·30	39·69	4·56
2·00–2·99	8·90	12	+3·10	9·61	1·10
3·00–3·99	7·59	7	−0·59	0·35	0·05
4·00–4·99	5·94	4	−1·94	3·76	0·63
5·00–5·99	4·41	2	−2·21	4·88	1·11
6·00–6·99	3·16	2	−1·16	1·35	0·43
7·00–8·99	3·74	1	−2·74	7·51	2·00
Over 9·0	3·05	1	−2·05	4·20	1·38
Total	50·00	50·00			11·76

$\chi^2 = 11{\cdot}76$; n = 8; P = 0·1–0·2

EXPERIMENTAL RESULTS

The Viability of Bacterium coli *in Distilled Water at* 20° C., *and the Release of Soluble Cell Constituents from the Organsims during Incubation*

Viable counts made at intervals on suspensions of *Bact. coli* stored at 20° C. in water from a heavily tinned still fitted with an all-glass condensing system are recorded in Table IV, and the corresponding log-time probit-mortality curve is shown in Figure 2.

The ultra-violet absorption spectra of cell-free supernatants from the bacterial suspension were examined at the time of making the viable counts and are plotted in Figure 3. Figure 4 shows the release of 260 mμ-absorbing material during storage of the cells in the distilled water, and

TABLE IV

VIABILITY OF *Bact. coli* IN DISTILLED WATER

Time (hours)	Log time	Survivors (per cent.)	Mortality (per cent.)	Probit mortality	Extinction 260 mμ
0	—	100	0	—	—
1	—	99·5	0·5	2·4242	0·07
24	1·3802	88·4	11·6	3·8048	0·117
100	2·0000	83·5	16·5	4·0259	0·158
187½	2·2730	73·4	26·6	4·3750	0·179
291½	2·4646	69·4	30·6	4·4928	0·195
528	2·7226	60·7	39·3	4·7285	0·246
695½	2·8423	58·6	41·4	4·7827	0·243
936	2·9713	55·1	44·9	4·8718	0·286
1345	3·1287	54·8	45·2	4·8794	0·326

Figure 5 the relation between the appearance of 260 mμ-absorbing material and the viability of the organisms in the distilled water.

Figure 5 shows that there is a gradual decline in the number of living organisms when *Bact. coli* is incubated at 20° C. in distilled water, and reveals that there is a simultaneous gradual appearance in the supernatant of material which has a maximal ultra-violet absorption at a wavelength of 260 mμ. The appearance of material having a similar ultra-violet absorption has previously been reported[14,15,16] in suspensions of bacteria in distilled water, 1 per cent. w/v sodium chloride solution and in phosphate buffer. Salton and Alexander[13] and Salton[14] have shown that free purines and pyrimidines are present in the released material.

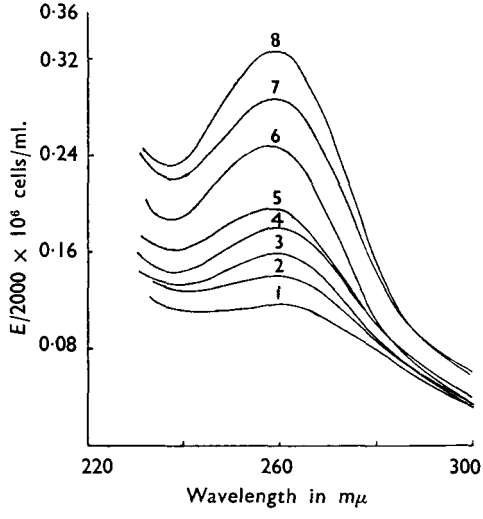

FIG. 3. Ultra-violet absorption spectra of cell-free supernatants from suspensions of *Bact. coli* in distilled water after varying contact times at 20° C. Curves 1 to 8 after 24, 48 100, 187·5, 291·5, 528, 936, 1345 hours respectively.

Salton[14] found that the amount of 260 mμ-absorbing material released from bacterial cells treated with cetrimide was proportional to the number of cells killed. Neither our own results nor those of Few and Schulman[16] support this finding. An examination of Figure 5, suggests that there may be a rectilinear relation between certain mortality ranges, but when the curve as a whole is examined no such rectilinear relation is apparent.

The Absorption of Benzylchlorophenol by Bact. coli

Suspensions of *Bact. coli* at a concentration of 2000 × 10^6 organisms per ml. were prepared in phosphate buffer solutions (*p*H 7·0) containing

10, 25, 50 and 100 μg. of benzylchlorophenol per ml. respectively. Immediately after preparation of the suspensions, the organisms were spun down at 8500 g. and the ultra-violet absorption at 282 mμ of the cell-free supernatant was examined. The extinction of the solutions before and after contact with the organisms is recorded in Table V where the "after contact" extinction has been corrected for the absorption at 282 mμ which is

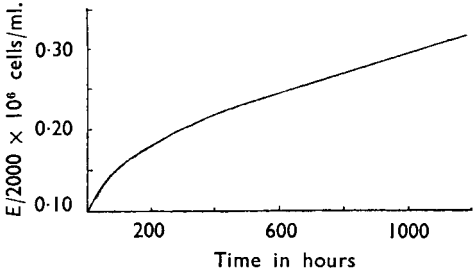

FIG. 4. Release of 260 mμ absorbing material from *Bact. coli* suspended in distilled water at 20° C.

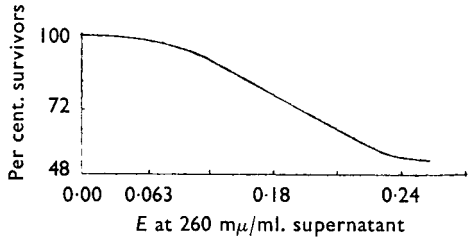

FIG. 5. Relationship between release of 260 mμ absorbing material and viability of *Bact. coli* suspended in distilled water at 20° C. over a period of 56 days.

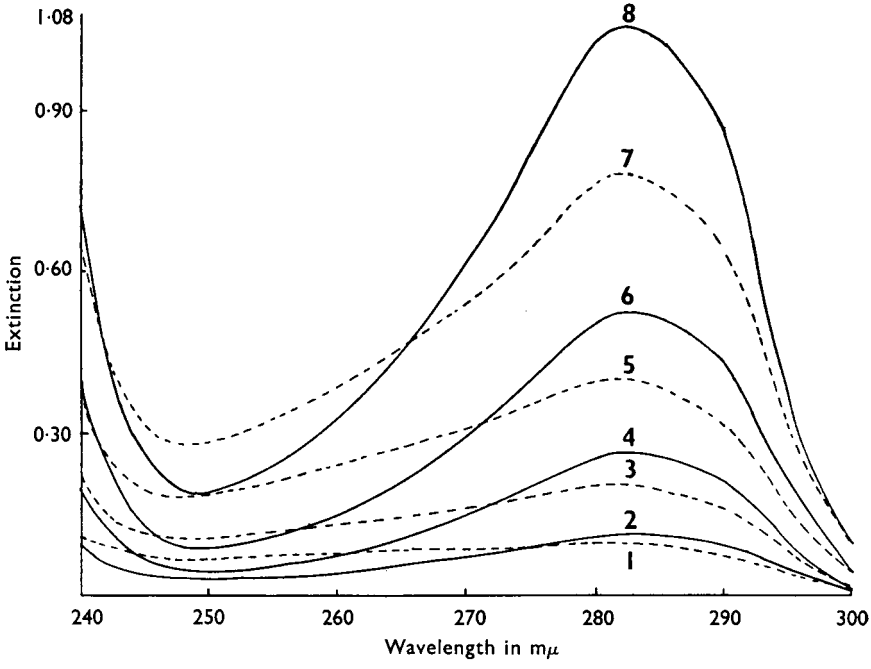

FIG. 6. Ultra-violet absorption spectra of benzylchlorophenol solutions (unbroken lines) and of the cell-free supernatants after contact with *Bact. coli* 2000 × 10^6 cells/ml. (broken lines).

1.	Supernatant from	10 μg./ml.	solution	2.	10 μg./ml. solution
3.	,,	,, 25 μg./ml.	,,	4.	25 μg./ml. ,,
5.	,,	,, 50 μg./ml.	,,	6.	50 μg./ml. ,,
7.	,,	,, 100 μg./ml.	,,	8.	100 μg./ml. ,,

TABLE V

EXTINCTION AT 282 mμ OF BENZYLCHLOROPHENOL SOLUTIONS BEFORE AND AFTER CONTACT WITH *Bact. coli*

Concentration of benzylchlorophenol (μg./ml.)	Extinction		Benzylchlorophenol remaining in supernatant (μg./ml.)	Benzylchlorophenol absorbed by *Bact. coli* (μg./ml.)
	Untreated solution	After contact with *Bact. coli*		
100	1·06	0·758	72	28
50	0·530	0·359	34	16
25	0·267	0·183	17·4	7·6
10	0·107	0·075	7·2	2·8

attributable to soluble cellular constituents released into the solutions. In these connections it is assumed that the cellular material released upon contact with the phenol is of a similar nature to that occurring when the cells are suspended in distilled water or phosphate buffer. Figure 6 shows the absorption spectra of the supernatants after immediate removal of *Bact. coli* together with the absorption spectra of the untreated solutions.

The absorption curve showing that the amount of benzylchlorophenol absorbed by the cells is proportional to the concentration in the supernatant is produced in Figure 7. The benzylchlorophenol absorbed

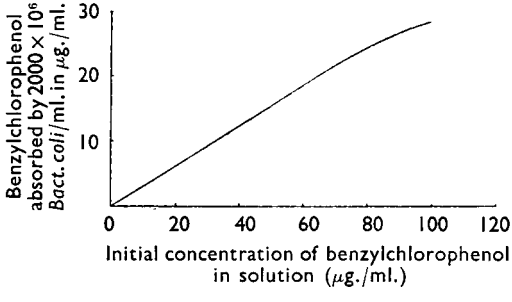

FIG. 7. Absorption of benzylchlorophenol by *Bact. coli* at 20° C.

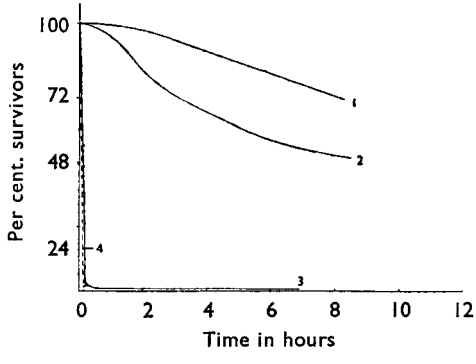

FIG. 8. Viability of *Bact. coli* in solutions of benzylchlorophenol in 0·01M phosphate buffer. Curves 1 to 3, 10, 25 and 50 μg./ml. respectively. Curve 4 (broken line) 100 μg./ml.

by the cells from the solutions at the 4 concentrations used viz. 10, 25, 50 and 100 μg. per ml. was 28, 30·4, 32 and 28 per cent. respectively of the amount initially present in the solutions. The shape of the absorption curve suggests that initial saturation of the organisms was not obtained in the concentrations used, and that greater quantities would have been taken up in the same time by the organisms from more concentrated solutions.

The Viability of Bact. coli *in Aqueous Solutions of Benzylchlorophenol*

Viable counts were made on suspensions of *Bact. coli* in 0·01M phosphate buffer solutions containing 10, 25, 50 and 100 μg. of benzylchlorophenol per ml. respectively. The percentage survivors at each contact time

examined are shown in Table VI, VII and VIII, and are plotted in Figure 8. In the solutions containing 10 and 25 μg. per ml. of benzylchlorophenol there was a steady decline in the number of survivors with time, and in the

TABLE VI

VIABILITY OF *Bact. coli* IN SOLUTIONS CONTAINING 10 AND 25 μG./ML. BENZYLCHLOROPHENOL AND THE EXTINCTION AT 260 AND 282 Mμ OF THE CELL-FREE SUPERNATANTS

Benzylchlorophenol concentration	Time (hours)	Survivors (per cent.)	Extinction	
			260 mμ	282 mμ
10 μg./ml.	0	100·0	0·071	0·093
	1	98·6	0·132	0·126
	2	95·3	—	—
	3	96·8	0·134	0·121
	4	89·2	—	—
	6	—	0·174	0·137
	6½	79·5	—	—
	8	72·5	0·194	0·143
25 μg./ml.	0	100·0	0·125	0·216
	1	95·7	0·169	0·226
	2½	79·6	0·172	0·223
	3	—	0·185	0·224
	4	67·2	0·194	0·228
	6	—	0·214	0·234
	6½	53·9	—	—
	7	—	0·227	0·237
	8	49·7	0·242	0·244
	24	35·5	0·405	0·297
	76	11·8	0·704	0·393

solution containing 100 μg. per ml. a very rapid decline in the number of survivors. The latter solution was sterile within 5 minutes. The solution containing 50 μg. per ml. of benzylchlorophenol is of special interest. In

TABLE VII

VIABILITY OF *Bact. coli* IN SOLUTIONS CONTAINING 50 μG./ML. BENZYLCHLOROPHENOL AND THE EXTINCTION AT 260 AND 282 Mμ OF THE CELL-FREE SUPERNATANTS

Experiment	Time	No. of viable organisms/ml.	Extinction	
			260 mμ	282 mμ
1	0	1,172,615,700	—	—
	5 min.	1,132,725	0·251	0·417
	1 hour	212,217	0·295	0·421
	4 ,,	20,437	0·356	0·421
	5 ,,	19,174	—	—
	5¾ ,,	19,290	—	—
	19 ,,	976,860	0·505	0·465
	26¼ ,,	5,858,240	0·521	0·473
	50½ ,,	5,010,480	0·629	0·507
	98¾ ,,	3,585,440	—	—
	124 ,,	3,544,330	0·859	0·587
	170½ ,,	9,682,970	—	—
	258¼ ,,	10,385,220	0·984	0·582
2	0	1,172,615,700	—	—
	5 min.	29,118,620	0·250	0·416
	2¾ hours	134,740	0·321	0·422
	4 ,,	82,100	—	—
	7 ,,	77,620	0·361	0·429
	8 ,,	108,430	—	—
	26½ ,,	1,287,160	0·492	0·467
	79 ,,	3,870,480	0·727	0·532
	103¼ ,,	3,542,600	—	—
	173¼ ,,	3,252,750	0·978	0·615
	317¼ ,,	4,283,550	—	—
	339¼ ,,	4,695,870	1·136	0·665
	653 ,,	2,267,760	1·291	0·742
	987 ,,	1,639,710	—	—

the 2 experiments reported in Table VII the percentage survivors after 5 to 7 hours is between 0·002 and 0·008. During the following few hundred hours a 50 to 500-fold increase in the number of living cells was observed. The causes of the variations in the several experiments performed with this concentration of benzylchlorophenol are under investigation, but it must nevertheless be acceded that the comparatively enormous multiplications in what was initially a bactericidal system are remarkable.

It does not appear that the observed multiplication can be accounted for by adaptation as normally understood, because washed cells were used and they were suspended in 0·01M phosphate buffer solution instead of a nutrient medium as normally used in adaptation studies.

TABLE VIII

VIABILITY OF *Bact. coli* IN SOLUTIONS OF BENZYLCHLOROPHENOL 100 μG. ML. AND THE EXTINCTION AT 260 AND 282 Mμ OF THE CELL-FREE SUPERNATANT

Time	Survivors (per cent.)	Extinction	
		260 mμ	282 mμ
0 min.	100	—	—
5 ,,	0	0·402	0·808
1 hour	0	0·420	0·810
5 ,,	0	0·476	0·814
8 ,,	0	0·470	0·808
26½ ,,	0	0·593	0·825
79½ ,,	0	0·868	0·884
128 ,,	0	1·011	0·946
272 ,,	0	1·174	0·952

The ultra-violet Absorption Spectra of Supernatants from Bact. coli *suspensions containing Benzylchlorophenol in 0·01M Phosphate Buffer Solution*

Two separate portions of the bacterial suspension were taken at each contact time. One was used for the viable count and the other was centrifuged at 8500 g. to provide a cell-free supernatant which was examined for absorption at 260 and 282 mμ. The absorptions are recorded in Tables VI, VII, VIII.

Figure 9 records the absorption spectra of supernatants after treatment of *Bact. coli* with solutions containing 50μg. per ml. of benzylchlorophenol in phosphate

TABLE IX

GROWTH OF *Bact. coli* AND THE EXTINCTION AT 260 Mμ IN CELL ELUATES OBTAINED

(*i*) *By storage of* Bact. coli *in distilled water for* 10 *weeks at* 20° *C.*

Time of incubation in cell eluate hours	Viable organisms (per ml.)	Log. viable organisms	Extinction at 260 mμ
0	205,980	5·3139	0·335
7½	209,280	5·3207	0·338
21⅓	2,985,960	6·4751	0·334
76	5,047,020	6·7030	0·333
102	4,951,100	6·6947	—
150	5,289,100	6·7233	—
340½	5,216,020	6·7173	0·345

(*ii*) *By heating a suspension of* Bact. coli *in distilled water at* 100° *C. for* 1 *hour.*

0	18,270	4·2617	1·132
6⅓	41,470	4·6167	—
23½	8,945,060	6·9515	1·122
78	19,048,580	7·2798	1·118
126	21,022,950	7·3226	1·109
146	22,413,360	7·3504	—
269½	22,580,210	7·3537	1·104
1011	18,229,900	7·2607	1·087

(*iii*) *By heating a suspension of* Bact. coli *in 0·01M phosphate buffer* (p*H* 7·0) *at* 100° *C. for* 1 *hour*

0	162	2·2095	0·913
3¾	222	2·3464	0·906
19	10,880	4·0370	0·910
72	18,817,840	7·2746	0·853
98	19,935,850	7·2997	0·854
331	18,561,737	7·2686	0·844

buffer for contact times to up 653 hours. The peak at 282 mμ in the absorption spectrum curve shows a sharp fall immediately after the addition of the organisms to the solution due to removal of benzylchlorophenol from the solutions by the bacterial cells. With continued incubation of the cells in the solution of benzylchlorophenol the extinction at 282 mμ gradually increased and ultimately became greater than the initial density due to the benzylchlorophenol. This increase in the amount of

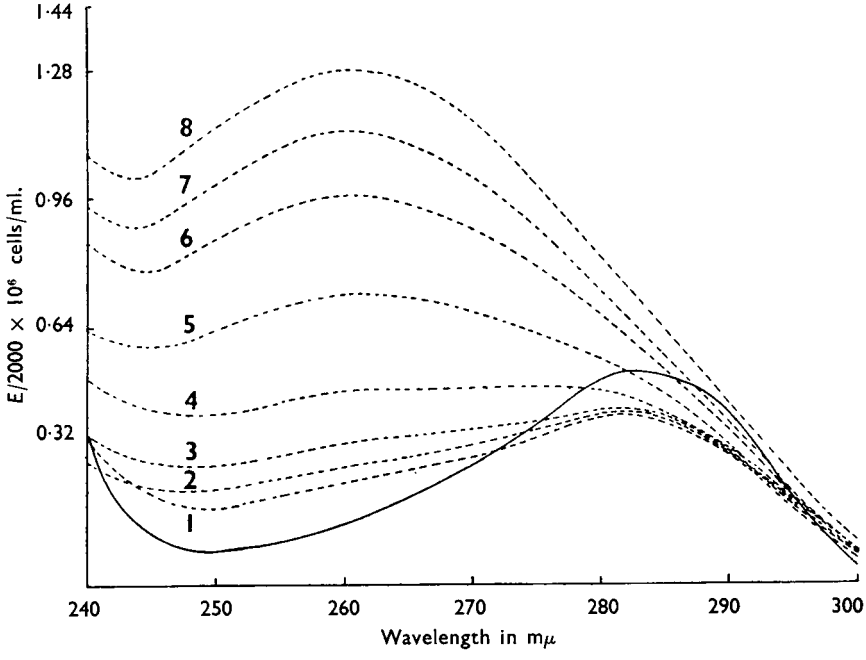

FIG. 9. Ultra-violet absorption spectra of supernatants from suspension of *Bact. coli* in solution of benzylchlorophenol 50 μg./ml. Curves 1 to 8 after zero time, 3, 7, 26·5, 79, 173·5, 339·5, 653 hours respectively. Unbroken line = untreated solution.

282 mμ-absorbing material can be attributed to material released from the bacterial cells upon treatment with benzylchlorophenol. The figure shows that simultaneously there was a marked increase in the absorption at 260 mμ due to material being released from damaged or killed bacterial cells. The release of 260 mμ-absorbing material was observed in each of the 4 concentrations of bactericide used, and in each concentration the extinction at 260 mμ was considerably higher than that obtained after similar time intervals from cells which had been suspended in distilled water. A similar pronounced absorption at 260 mμ has been noted in the supernatants of cells treated with polymyxin E and cetrimide[13,14,15,16].

Figure 10 shows that 260 mμ-absorbing material continues to be released over many days from cells treated with benzylchlorophenol even though all the cells are killed within a few minutes. This supports the suggestion made earlier in this paper that there is apparently no simple relation

between the mortality of *Bact. coli* and the amount of 260 mμ-absorbing material released from the cells.

The Viability of Bact. coli *in cell-eluates*

In view of the multiplication previously described when *Bact. coli* was treated with benzylchlorophenol 50 μg/ml. the possibility of cellular release materials functioning as a nutrient material was investigated. The cell-eluate was obtained by storage of *Bact. coli* in distilled water and also by maintaining at a temperature of 100° C. for 1 hour.

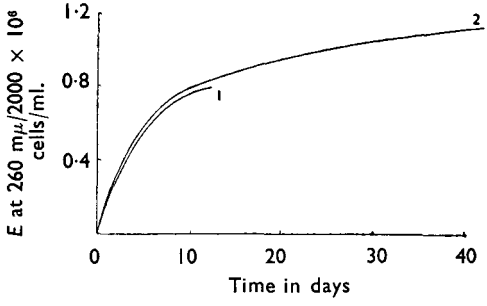

FIG. 10. Release of 260 mμ absorbing material from *Bact. coli* during incubation in benzylchlorophenol solution at 20° C. Curve 1 = 100 μg./ml., curve 2 = 50 μg./ml.

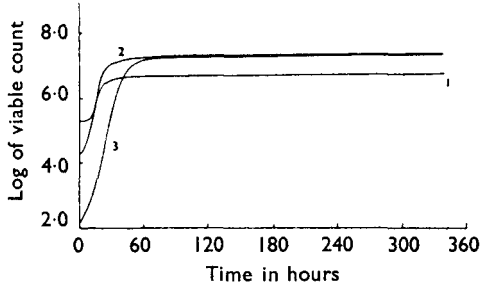

FIG. 11. Multiplication of *Bact. coli* in cell eluates.
Curve 1. Eluate obtained by storage in distilled water.
Curves 2 and 3. Eluate obtained by heating suspension of *Bact. coli* in distilled water and phosphate buffer for 1 hour respectively.

(*a*) *Cell-eluate obtained by storage*

A suspension of *Bact. coli* was prepared as usual in distilled water and left for 10 weeks at 20° C. The majority of the cells were then removed by centrifuging at 8500 g. for 3 minutes and the supernatant filtered through a 5 on 3 sintered glass filter. One ml. of the filtrate transferred to 10 ml. of nutrient broth showed no growth on incubation.

A new suspension of *Bact. coli* was prepared in distilled water and a small inoculum was added to the cell-eluate.

(*b*) *Cell-eluate obtained by heating at* 100° *C. for* 1 *hour*

Suspensions of *Bact. coli* in distilled water and 0·01M phosphate buffer were prepared by the usual method, and then heated at 100° C. for 1 hour. The bacteria were removed and the cell-free supernatants were inoculated with a freshly prepared suspension of *Bact. coli* in distilled water and 0·01M phosphate buffer respectively and incubated at 20° C. The number of viable organisms and the ultra-violet absorption spectra of the cell-free supernatants were determined at various time-intervals. Table IX tabulates the numbers of viable organisms and the ultra-violet absorption at 260 mμ, and Figure 11 relates the log of the viable count to the time of incubation.

Discussion

The results obtained indicate that materials released into the suspending fluids when *Bact. coli* die are capable of promoting cell-growth. When a small inoculum of organisms is added to the cell-eluate and incubated, a typical growth curve is obtained. It is thus possible that the leakage of material from *Bact. coli* when treated with benzylchlorophenol 50 μg./ml., is a contributory factor in the multiplication of the surviving organisms. Similarly, the reduction in mortality rate with time, of many organisms when treated with bactericides, which is frequently observed at the distal portions of the survivor-time curves, and which has been attributed to an increased resistance of the last survivors, may be in part due to cellular release materials in sub-optimal nutrient concentrations.

A recent paper by Whitehead[25], demonstrated that when a suspension of *Bact. coli* was irradiated by ultra-violet light and the cells removed, washed, and re-suspended in the original suspending fluid or in fresh phosphate buffer, the mortality of the cells returned to the original suspending fluid was only 25 per cent. of that of cells suspended in fresh buffer. Whitehead suggested that a "restorative factor" was released by the cells as a physiological response to injury of the cells by the irradiation. It would appear that his observation is analogous to that described in the present communication.

The change in ultra-violet absorption at 260 mμ shown in Table IX, which takes place simultaneously with the multiplication of the organisms is surprisingly small for the amount of growth observed. The significance of this observation is being further investigated.

Summary

1. The absorption by *Bact. coli* of benzylchlorophenol from aqueous solution has been demonstrated and an absorption isotherm plotted.

2. Soluble cellular constituents which have a maximal ultra-violet absorption at 260 mμ are released when *Bact. coli* dies in aqueous solutions of benzylchlorophenol.

3. Multiplication of *Bact. coli* survivors has been observed in a solution of benzylchlorophenol demonstrated to be initially bactericidal.

4. Water-soluble cellular constituents extracted from *Bact. coli* are capable of promoting cell growth.

5. It is suggested that soluble cellular constituents released when *Bact. coli* dies in aqueous solutions of benzylchlorophenol can influence the viability of the last survivors and alter the course of the bactericidal reaction.

The authors wish to acknowledge a generous gift of benzylchlorophenol by the Cocker Chemical Co. Ltd.; they are also indebted to Mr. G. O. Jolliffe for advice and assistance in the use of the spectrophotometer.

References

1. Madsen and Nyman, *Z. Hyg. InfektKr.*, 1907, **57**, 388.
2. Chick, *J. Hyg., Camb.*, 1910, **10**, 237.
3. Rahn, *Injury and Death of Bacteria by Chemical Agents*, Biodynamica, Normandy, Missouri, 1945.

4. Hewlett, *Digest on Disinfection*, Biological Research Ltd., 1936.
5. Reichel, *Biochem. Z.*, 1909, **22**, 149.
6. Reichenbach, *Z. Hyg. InfektKr.*, 1911, **69**, 171.
7. Henderson Smith, *Ann. appl. Biol.*, 1921, **8**, 27.
8. Henderson Smith, *ibid.*, 1923, **10**, 335.
9. Withell, *J. Hyg., Camb.*, 1942, **42**, 124.
10. Berry and Michaels, *Quart. J. Pharm. Pharmacol.*, 1947, **20**, 348.
11. Berry and Michaels, *J. Pharm. Pharmacol.*, 1950, **2**, 382.
12. Jordan and Jacobs, *J. Hyg., Camb.*, 1944, **43**, 275.
13. Salton and Alexander, *J. gen. Microbiol.*, 1950, **4**, ii.
14. Salton, *ibid.*, 1951, **5**, 391.
15. Newton, *ibid.*, 1953, **9**, 54.
16. Few and Schulman, *ibid.*, 1953, **9**, 454.
17. Berry and Michaels, *Quart. J. Pharm. Pharmacol.*, 1947, **20**, 331.
18. Berry and Bean, *J. Pharm. Pharmacol.*, 1951, **3**, 639.
19. Allawala and Riegelman, *J. Amer. pharm. Ass., Sci. Ed.*, 1953, **42**, 267.
20. Wilson, *J. Bact.*, 1922, **7**, 405.
21. Withell, *Quart. J. Pharm. Pharmacol.*, 1938, **11**, 736.
22. Bullock, Keepe and Rawlins, *J. Pharm. Pharmacol.*, 1949, **1**, 878.
23. Wilson, *The Bacteriological Grading of Milk, Spec. Rep. Ser. med. Res. Coun., Lond.*, 1935, No. 206.
24. Thornton, *Ann. app. Biol.*, 1922, **9**, 241.
25. Whitehead, *Can. J. Microbiol.*, 1955, **1**, 266.

DISCUSSION

The paper was presented by MR. V. WALTERS.

DR. K. R. CAPPER (London) said it would be interesting to learn something about the character of the survivors. That material which would support the growth of the organism could be released from cells was perhaps not at all surprising, but the fact that growth occurred in the presence of quantities of a phenolic substance seemed to indicate that the survivors had characteristics which were not shared by the culture as a whole. In many cultures there were organisms with abnormal resistance, but they usually had cultural characters which differed from those of the original organisms. Had any work been done on the survivors to find out whether that resistance could be inherited, and whether they were different in cultural requirements from the original *Bact. coli* strain? In any subsequent work carried out on the material absorbing at 260 mμ it would be better if a synthetic medium were used.

MR. G. SYKES (Nottingham) said he was disappointed to find that the references did not include the name of Gale because of his fundamental work on the role of glutamic acid and the prevention of the diffusion of that substance in the presence of certain surface-active agents. He did not believe that this phenomenon could be anything more than partially contributory to the problem described, because there was always a residual excessive resistance even with a relatively small bacterial population, when the concentration of the nutritive material released into the solution must be infinitely small. He also found it difficult to associate the phenomenon with the well known phenomenon of adaptation.

MR. H. D. C. RAPSON (Dorking) referred to the experimental results discussed under the heading "Absorption of Benzylchlorophenol by *Bact. coli*" and the relevant data of Fig. 7, which was presented in the form of an "isotherm" it being stated that adsorption was proportional to the

concentration. The "isotherm" of Fig. 7 was curved, consequently the amount adsorbed was not strictly proportional to the concentration. Although that did not seriously affect the substance of the paper, it tended to inhibit a fuller appraisal of the result. Since the term "isotherm" was used, the data should have been presented in a more conventional manner, that is, the amount adsorbed should have been plotted against the concentration of the solution in contact with the surface. If that procedure were adopted, the curvature would be found to be more pronounced, and to start in the 50 μg./ml. region which was interesting in view of the anomalous results. If the isotherm concept were extended and the approximate surface area of the bacterium were calculated, it could be shown that something approximating to a monomolecular layer of bactericide was present on the surface of the bacterium, suggesting that the organism might have died of "asphyxiation". It was very desirable to treat the results of such calculations with caution since a living organism was involved. If on expiration the effective area of the bacterium were suddenly to increase, then a physical explanation of the abnormal viability could be given. Adsorption techniques afforded a powerful method whereby surfaces might be studied and it should be possible thereby to differentiate between the lethal effect due to processes connected with adsorption, and that following diffusion into the organism.

DR. G. E. FOSTER (Dartford) asked the authors whether they could give further details of the chemical nature of the material absorbing at 260 mμ.

DR. A. H. BECKETT (Chelsea) said that the measurement of the absorption of phenols in the presence of ultra-violet absorbing materials released from the bacteria at present must only be looked upon as very approximate.

MR. V. WALTERS, in reply, said that they had not determined whether the residual viable organisms had developed a resistance. He agreed that the name of Gale should have been included in the references and that the absorption curve shown in the paper should have been expressed as had been suggested and not as an initial concentration. He had no personal knowledge of the chemical nature of the substances liberated though he had commenced some paper chromatographic work to elucidate the amino-acids. He could only quote from previous work that they consisted of purines and pyrimidines. With regard to the measurement of the phenol absorption, the Morton-Stubbs correction had been applied to calculate the amount of phenol absorbed, and bearing in mind the 260 mμ fraction also absorbed at 280 mμ, the values obtained for the percentage absorption using that correction were respectively: 38·5, 37, 40 and 40, roughly 10 per cent. higher. Unfortunately, it did not follow that the Morton-Stubbs correction was applicable since the absorption contribution of cellular released material appeared to be as much at 280 mμ as at 260 mμ.