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Some Factors affecting the Destruction of *Bacterium coli* by Shaking with Glass Beads

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SUMMARY: *Bacterium coli* was killed rapidly when shaken with glass beads. The bacteria were killed before they were disintegrated. The effect on this process of size of bead, speed of shaking, density of suspension, and size of container were investigated and optimal conditions for bacterial destruction were ascertained.

Curran & Evans (1942) destroyed various bacterial spores and *Bacterium coli* cells by shaking in suspension with small inert particles. They studied the effect of different kinds of particles, the proportion of these to spores and density of the spore suspension. They noted in one experiment that efficiency increased with speed of shaking.

King & Alexander (1948) amplified these observations, using glass beads, and a wider range of sporing and non-sporing bacteria. They confirmed that the optimal effect was obtained when the weight of beads in grams was the same as the volume of suspension in millilitres and that the rate of bacterial destruction increased with greater speed of shaking. They were, however, unable to sterilize a suspension of *Bact. coli*, as reported by Curran & Evans, but found that there was a proportion of all bacteria tested which was resistant to destruction by shaking with glass beads. The proportion with their strain of *Bact. coli* was 1 in 100,000. They expressed the rate of destruction as 'decimal reduction time' (D.R.T.)—time required to reduce the viable count to 1/10 of the original.

The investigations reported here were made to assess the relative importance of speed of shaking, size of bead, size of container and density of suspension in reducing the viable count and in disintegrating the bacteria, and to determine the most effective combination of these factors.

METHODS

The shaker had adjustable set speeds between 430 and 700 double horizontal excursions or shakes/minute (s.p.m.) at an amplitude of 31 mm. Four bottles could be shaken at once which facilitated comparative tests. Airflow over the containers prevented undue rise in temperature.

Glass beads (Messrs Chance Bros. Ltd., Smethwick)—Ballotini grades 9, 12 and 16—were used. They had mean diameters of 337, 171 and 107 μ ., respectively, based on the measurement of 100 beads. The distribution of sizes in each grade is shown in Table 1. Before use they were soaked in chromic-sulphuric acid mixture, thoroughly washed in water, dried and sterilized in the hot-air oven. Twenty-five g. were added to 25 ml. of suspension.

Table 1. *The distribution of diameters of glass beads within each grade*

Grade 9		Grade 12		Grade 16	
Diameter (μ .)	%	Diameter (μ .)	%	Diameter (μ .)	%
< 260	3	< 100	5	< 80	8
261-280	10	101-120	9	81-90	6
281-300	13	121-140	15	91-100	17
301-320	18	141-160	16	101-110	24
321-340	11	161-180	15	111-120	23
341-360	12	181-200	16	121-130	14
361-380	14	201-220	13	131-140	8
381-400	15	221-240	9	> 140	0
> 400	4	> 240	2	—	—

Three sizes of bottle were used: (a) a screw-capped '10 oz. flat' with the neck cut off about 12 mm. from the shoulder, holding 300 ml. It was placed on its narrow side; the internal distance between the sides, in the line of excursion, was about 30 mm.; (b) a bottle with square base, internal distance between sides about 30 mm., 11.2 cm. to the shoulder, holding 130 ml. It was placed upright; (c) a square-based bottle, internal distance between sides about 50 mm., 13 cm. to the shoulder, holding 420 ml. placed on its side, transversely to the line of excursion. The bottles were stoppered with pressure tubing dilated to the correct size with a glass rod and inserted to the level of the shoulder of the bottle. There was always some suspension in a thin layer around the stopper which, when the stopper was removed after shaking, mixed with the contents of the bottle, and by adding living bacteria, introduced an error in estimating the decrease of viable counts caused by shaking, especially when the contents had been nearly sterilized.

Suspensions of *Bact. coli* were made in distilled water from agar plate cultures incubated at 37° for 18 hr.; they were equal in opacity to Brown's no. 1 tube (Burroughs, Wellcome and Co.) and contained about 800×10^6 organisms/ml. To break up clumps of cells, 100 ml. of suspension was shaken in bottle (a) at 600 s.p.m. for 10 min. This appeared to be the optimal time; shaking for a further 15 min. decreased the viable count by 5-6%. A similar suspension kept at room temperature for 4 hr. showed an increase in the viable count from 620 to 870×10^6 . The conditions of suspension were therefore not deleterious.

Viable counts were made in duplicate by placing 1 ml. of suitable dilution in a Petri dish, mixing with 15 ml. agar, melted and cooled to 50°, incubating for 24 hr. at 37° and counting with a $\times 2\frac{1}{2}$ lens. Incubation for 48 hr. did not increase the count.

After breaking up the clumps, 25 ml. of suspension was transferred to a bottle for experimental shaking. The logarithm of the number of organisms surviving after several periods of shaking was plotted and from the graph the D.R.T. ascertained. This procedure facilitated the comparison of results in successive experiments with suspensions containing different numbers of organisms.

Factors affecting the rate of killing of Bacterium coli

Shaking alone. Shaking without beads for 3 hr. at 530, 600 and 700 s.p.m. decreased the viable count by 16·5, 20·7 and 19·5 % respectively. Similar results were obtained with each size of bottle. This diminution was small compared with shaking with beads.

Size of bead. The addition of beads of any of the sizes tested shortened the D.R.T. markedly as compared with shaking alone. With each speed of shaking, decreasing the size of the beads shortened the D.R.T. (Table 2). When the logarithm of the number of surviving organisms was plotted against the time shaken, the decrease followed the exponential law until the viable count reached a figure between log 2 and log 3, after which there was a decline in the rate of destruction. On plotting the D.R.T. against mean diameter of bead for each speed separately, the points fell in each case on a straight line, indicating a linear relationship. Thus for any beads having a mean diameter within the range 107–337 μ , the rate of destruction can be calculated. Beads with a still smaller mean diameter were not obtainable, but it seems probable that further decrease in size would have reached a limit of increasing effectiveness.

Table 2. *The effect of size of bead and speed of shaking on the killing of Bacterium coli*

Shakes per min.	Bead grade 9 Type of bottle			Bead grade 12 Type of bottle			Bead grade 16 Type of bottle		
	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
	D.R.T. (time in min. to reduce count by 90 %)								
530	46	47	47	21·5	21	23	13	13	12
600	27	27	27	13	12	13	6	6·5	6
700	26	25	27	8·5	9	9	3·5	3·5	3·5

Density of cell suspension. Suspensions of different densities were shaken simultaneously at 600 s.p.m. with grade sixteen beads. The D.R.T. of suspensions up to 1335×10^6 cells/ml. was 6 min. Increasing this density tenfold prolonged the D.R.T. to 10 min.

Speed of shaking. Increase in speed between 530 and 700 s.p.m. shortened the D.R.T. for two of the three grades of beads tested (Table 3); grade 9 exerted its maximum efficiency at 600 s.p.m., the slight difference at 700 s.p.m. being within experimental error.

Size of container. The amount of suspension was always 25 ml. which, stated as a proportion of the volume of bottles (a), (b) and (c) respectively, was 1 : 12, 1 : 5·2 and 1 : 16·8. The distance between the sides of the bottles (a) and (b), in the direction of the movement, was the same as the amplitude of the excursion, for bottle (c) it was nearly twice as great. The results (Table 2) indicate, however, that the differences in the three bottles used had no significant effect on the result as similar results were obtained with all bottles for each size of bead.

A comparison of total and viable counts

Comparisons were made of total counts, using a counting chamber, and viable counts using pour plates prepared from the same dilutions. In every case the total count was higher than the viable count, indicating that the beads first killed the organisms without apparent injury as viewed in stained films, by dark ground illumination, and in hanging-drop preparations; disintegration followed later. Differences between the two counts depended on the size of the bead. The rate of destruction followed the exponential law. With grade 9 beads the ratio of total to viable counts averaged 6 : 1 or 8 : 1 at 600 s.p.m.; with the smaller beads the ratio was greater. With decreasing sizes of beads the killing increased faster than the disintegrating effect.

Effect of prolonged shaking with beads

Attempts were made to sterilize suspensions of *Bact. coli* by prolonged shaking (Table 3). Complete sterilization was not obtained, but the numbers of viable organisms that remained were very small. In Exp. 3 a suspension

Table 3. *The destruction of Bacterium coli by prolonged shaking with beads*

Exp. no.	Shakes per min.	Bead grade	Time shaken (hr.)	Viable count (organisms/ml.)	
				Before shaking	After shaking
1	530	12	3	15×10^6	125
2	530	16	3	1070×10^6	24
3	600	12	3	$63,200 \times 10^6$	3.5
4	600	16	4	450×10^6	2.5
5	600	16	$1\frac{1}{2}$	630×10^6	9.5
6	600	16	$1\frac{1}{2}$	545×10^6	10.5

containing $63,200 \times 10^6$ organisms/ml. after 3 hr. shaking with grade 12 beads at 600 s.p.m. contained only 3–4 organisms/ml. In Exps. 4 and 6 the bottle was stoppered with pressure tubing which had been dilated to the correct size with glass tubing and the whole covered with a rubber membrane. The bottle was sampled once after shaking. A hypodermic needle was inserted through the glass tube, the membrane pierced, and several ml. of the suspension withdrawn. Although this method avoided removing the bung and consequent liberation of bacteria which had been protected from the beads in films and crevices around the bung, there was the possibility that the few remaining viable bacteria had escaped destruction in such situations; alternatively they might represent the last few bacteria which only very prolonged shaking would eradicate.

DISCUSSION

Although the results were not affected by variations in size of container and by the proportion of the volume occupied by suspension, the bottles were never more than one-fifth full and the distance between the vertical sides was never less than the excursion of the shake or greater than twice the

distance. It is therefore possible that nearly full bottles and bottles outside these limits might influence the results.

The limit of effectiveness of speed of shaking was reached only with the larger beads, grade 9. There would no doubt be a limit to the increasing efficiency of higher speeds of shaking and of smaller beads but for technical reasons these have not been investigated. With our strain of *Bact. coli*, however, the number of viable organisms was decreased to as few as 2·5 organisms/ml. after 4 hr. shaking and there does not appear to be a percentage of resistant cells as reported by King & Alexander (1948).

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