THE KILLING OF BACTERIAL SPORES IN FLUIDS BY AGITATION WITH SMALL INERT PARTICLES

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Among the mechanical agencies capable of killing bacteria, one which has passed almost unnoticed is that involved in the rapid shaking of bacterial suspensions with small, hard, inert particles. If such action is continued for some time the cumulative result of abrasion and repeated collisions between the particles and the bacteria is a disintegration of the latter.

The first and apparently the only investigator to describe this phenomenon was Meltzer (1892, 1894), who observed that certain vegetative bacteria contained in broth or salt solution were killed when subjected to long and vigorous mechanical shaking in contact with finely-divided materials such as zinc filings, sand, glass, and steel. The bacteria were not uniformly affected and, according to the data submitted, one species was greatly stimulated in its development even when agitation was continued without interruption for 8 days.

Our attention was directed to this subject by a fortuitous observation: A culture of spores was encountered which consistently yielded a higher plate count after it had been heated at 85°C. for 10 minutes. Further study revealed that this degree of heat effected the dispersion of a number of small spore clusters which in turn increased the number of colony foci. In an effort to obtain complete dispersion of these clumps by other means the suspension was subjected to prolonged mechanical agitation in the presence of sterile sand. The marked sporicidal action of this procedure became apparent when the suspension was replated.

In the following paper an effort has been made to evaluate some of the factors that influence the germicidal efficiency of this method.

METHODS AND MATERIALS

The principal test organisms were *Bacillus cohaerens* (A. T. C. C.) and *Bacillus megatherium* (N. R. Smith #234). Four other cultures were used for single experiments: *Bacillus subtilis* (A. T. C. C. #6051), two unidentified species #9499 and #1518 (National Canners' Association) and *Escherichia coli* (R. P. Tittsler #222). The method of cultivation and preparation of the test spores was similar to that previously described (Curran and Evans, 1937). The stock suspensions were stored at 6°C. All of the suspensions were 100 per cent spores except those of #1518, whose vegetative cells were killed by heat at 85°C for 10 minutes prior to use. The resistance of the spores both to heat and mechanical destruction remained practically unchanged during the period of study.

The shaking was done mechanically in an apparatus of the type commonly employed in sero-vaccine work. This provides a reciprocating up and down and slightly elliptical motion. Except where otherwise noted, the speed of the shaft was 430 r.p.m., which also represents the number of complete shakes per minute. The effective amplitude of the stroke was approximately $1\frac{3}{8}$ inches. The samples, 25 ml. in volume, were contained in 8-ounce screw-top prescription bottles held in an upright position. The capacity of the apparatus is 8 of these bottles, but for this work only those positions equidistant from the central shaft were used. Two defects in the design of the sample bottles should be mentioned: the slightly tapering neck affords a somewhat protective pocket, while the possibility of very slight seepage into the spaces around the threaded cap is never entirely excluded.

Before use, the abrasives were sifted, washed with flowing hot water for about 60 minutes, rinsed with distilled water, dried and weighed into sample bottles. Unless otherwise stated, 20

g. of abrasive was used. The sand (sea) particles were those passing a number 40 sieve; the glass beads (glow beads) 60/80 grade, i.e., passing a number 60 but retained by a number 80 sieve. The mere presence of abrasives in the cell suspensions had no effect upon the viability of the organisms used. Certain of the abrasives, notably carborundum, boron carbide, emery, and sand, when long and vigorously shaken in fluids, undergo a slight but progressive breakdown with the formation of a relatively stable suspension of finely divided material. This introduces a small but relatively insignificant error in plating.

Neither sand nor glass produced significant changes in pH when unbuffered suspensions were shaken for long periods and such treatment caused no appreciable change in temperature.

The number of colonies that developed on glucose extract agar plates before and after treatment was regarded as a measure of the test influence. The colonies were counted after the plates were incubated for 48 hours at the optimum temperature of the organism. At infrequent intervals the plates were returned to the incubator and reexamined after several additional days of incubation.

EXPERIMENTAL

When a uniformly dispersed suspension of bacterial spores is vigorously and evenly shaken with small glass beads the organisms die at the rate shown in figure 1. The logarithms of the number of surviving spores plotted against time fall along a descending straight line indicating that the death rate has the characteristics of a unimolecular reaction. Substitution of sand or other angular particles for glass beads alters the slope but not the essential nature of this curve. Plate 1 shows the visible changes produced in spores by long-continued shaking with glass beads. At the end of five hours hardly any spores remain intact (PM 2); the irregular stained material represents spore fragments in various stages of disintegration. After 15 hours' shaking, (PM 3) fragments are still discernible but in a more finely divided state.

The opacity of suspensions so shaken changes in a rather striking manner. In table 1 are recorded the light transmission values for the spore suspension before and after treatment. As

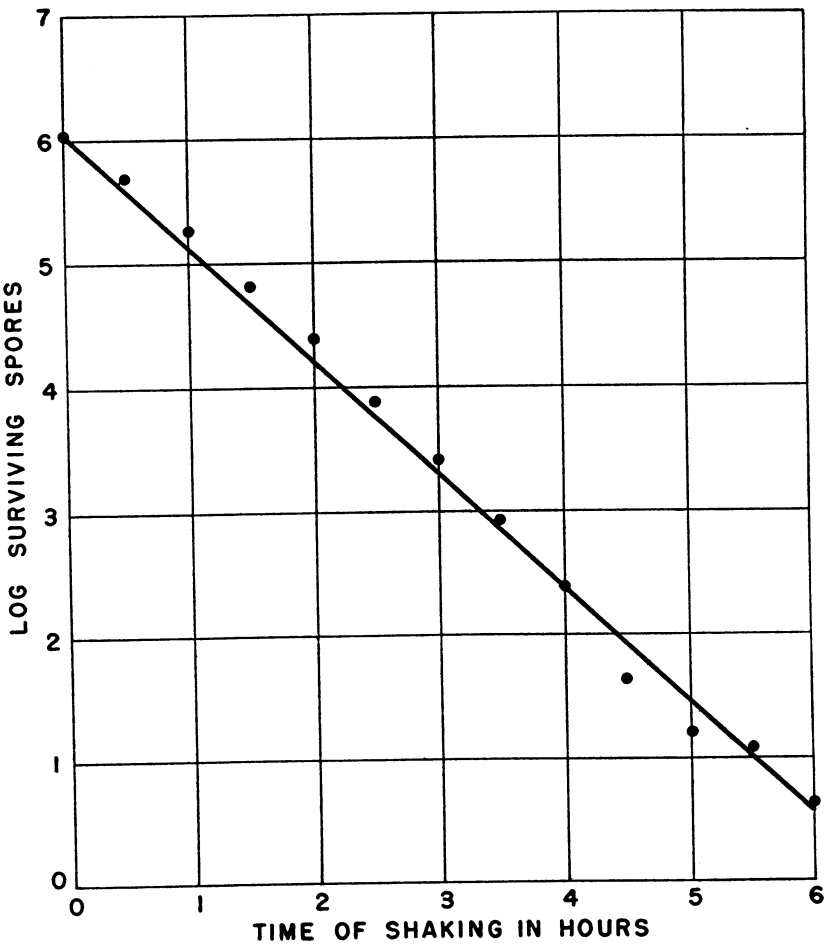


FIG. 1. THE KILLING OF B. SUBTILIS SPORES IN WATER WHEN SHAKEN FOR DIFFERING PERIODS WITH GLASS BEADS
25 ml. of spore suspension, 20 g. of glass beads 60/80 grade

may be seen (table 1) five hours' shaking with beads increased the light transmission of the suspension by about 23 per cent, but 11 hours' additional shaking only slightly increased the 5-hour

value. The changes in opacity of the shaken suspensions indicate that much of the spore substance is reduced to very small particles. A similar change in sonically killed bacteria has been reported by Chambers and Flosdorf (1936).

TABLE 1

The opacity of a spore suspension as affected by shaking with small glass beads†*

PERIOD OF SHAKING	LIGHT TRANSMISSION‡
hours	*per cent*
0	8.5
5	31.6
16	37.4

* *B. subtilis.*

† 30 gm. glass beads (sieves 60/80) used with 25 ml. of a suspension of the spores in distilled water.

‡ As determined by aminco Type "F" Photometer.

Action of different abrasives

In table 2 is shown the action of several abrasives upon *B. cohaerens* and *B. megatherium* spores. The criterion of germicidal activity was the number of spores that survived five hours of shaking. Two groups of materials were compared, one in which the particles were rather coarse (sieves 20/40), the other in which comparatively small particles composed the abrasive (sieves 80/100); the suspensions were buffered, since some of the test substances produced considerable alkalinity when unbuffered suspensions were shaken for long periods. In the group made up of the coarser particles, Pyrex chips were most effective, glass beads next, followed by boron carbide, sand, and carborundum. In the second group (lower part of table) the remarkable efficiency of the glass beads stands out. Where the angular particles were concerned, carborundum was first in effectiveness, followed by boron carbide, emery, and alundum. Adsorption of the spores upon the abrasive particles did not occur.

Some of the physical characteristics of the abrasives (Tone, 1939) are listed in this table. Boron carbide is the hardest known substance except diamond, carborundum next, and alundum ranks about fourth in hardness. Among those comprising the

coarser group, Pyrex chips, the softest and one of the lightest, was most sporicidal. When the particles were smaller, the smooth, spherical but comparatively soft glass beads were much more effective than were any of the substances composed of angular particles. Great importance cannot therefore be attached to the degree of hardness of the abrasive.

TABLE 2

The action of different abrasives when shaken with bacterial spores suspended in buffer solution (pH 7.0)

ABRASIVE	PARTICLE SIZE*	SPECIFIC GRAVITY	HARDNESS, MOHR'S SCALE	CRYSTALLINE FORM	VIABLE SPORES AFTER 5 HOURS' SHAKING	
					B. cohaerens†	*B. megatherium*‡
					per ml.	*per ml.*
Sand	20/40	2.5–2.6	7	RH and H§	70,000	7,300
Pyrex chips	20/40	2.6–2.8	5.5–6.5		39,800	750
Carborundum (Sic)	20/40	3.1–3.2	9+	T¶	95,500	8,050
Boron carbide (B_4C)	20/40	2.3–2.6	9+	RH and H	44,000	5,400
Glass beads	20/40		5.5		42,800	2,700
Alundum (Al_2O_3 fused)	80/100	3.9–4.0	9+	T	172,000	30,200
Emery ($Al_2O_3Fe_3O_4$)	80/100	3.8–4.0	7–9	RH and H	173,000	20,800
Carborundum (Sic)	80/100	3.1–3.2	9+	T	7,900	300
Boron carbide (B_4C)	80/100	2.3–2.6	9+	RH and H	120,000	17,400
Glass beads	80/100		5.5		30	3

* Refers to sieve numbers; i.e., 20/40 = through sieve 20, not through sieve 40.
† At 0 hr. 1,350,000 per ml.
‡ At 0 hr. 950,000 per ml.
§ Rhombohedral and hexagonal.
¶ Trigonal.

The importance of crystalline form cannot be appraised from these data; however, it is interesting to note that alundum and carborundum, both of extreme hardness and comparable in crystal structure, differed widely in their effectiveness. The greater specific gravity of the former was perhaps a factor in producing these differences.

Particle size

The rate at which fluid suspensions of spores are killed by moving abrasives is influenced by the size of the component

particles. Table 3 shows the results obtained with shaking procedures when particles of graded sizes were used. Carborun-

TABLE 3

The action of abrasives of differing particle sizes when shaken at 460 s.p.m. with spores suspended in water

PARTICLE SIZE	VIABLE SPORES AFTER 5 HOURS' SHAKING	
	B. cohaerens	*B. megatherium*
	per ml.	*per ml.*
No abrasive	1,170,000	730,000
Carborundum (Abrasive number)		
20	290,000	
30*	148,000	
40	18,100	2,500
50	6,400	600
60	4,850	250
70	4,100	150
80†	3,850	150
90	5,700	300
100	27,500	3,200
120	83,000	11,100
No abrasive	1,280,000	760,000
Sand (Sieve numbers)		
10	1,110,000	
10/20	500,000	350,000
20/40	117,000	17,500
40/60	17,000	1,100
60/80	36,000	8,300
No abrasive	1,280,000	760,000
Glass beads (Sieve numbers)		
20/40	131,000	2,320
40/60	37	18
60/80	13	1
80/100	30	3

* Equal to 20/40 sieve numbers.

† Approx. equal to 80/100 sieve numbers.

dum numbers refer to established designations used by abrasive manufacturers. The particle size decreases as the numbers increase. As may be seen (table 3) carborundum #80 was most

effective for the spores of both species. When the particles were smaller than this, despite a large increase in their number, the sporicidal activity was greatly reduced. Larger and fewer particles were also less effective. With sand as the abrasive the 40/60 grade was most effective. This was found to be true for samples obtained from different sources, although their relative effectiveness is subject to considerable variation. Glass beads, 60/80 grade, were most effective. Except for the coarsest grade, the differences were not large. This experiment indicates that the most effective particle size differs with the abrasive.

TABLE 4

The destruction of spores in water as influenced by the quantities of abrasive with which they are shaken*

WEIGHT OF ABRASIVE	VIABLE SPORES AFTER 5 HOURS' SHAKING		
	B. cohaerens		*B. megatherium*
	Sand	Glass beads	Sand
grams	*per ml.*	*per ml.*	*per ml.*
0	1,060,000	1,170,000	930,000
15	165,000	115	15,500
20	71,000	24	2,700
25	24,000	9	1,310
30	9,100	10	150
35	7,600	7	20
40	2,400	8	10
45	1,900	10	10
50	2,900	24	40

* 25 ml. of spore suspension used.

Quantity of abrasive

Since, in shaking procedures the quantity of abrasive used largely determines the number of striking particles, the germicidal activity of such procedures must necessarily be influenced by this factor. Data bearing on this point are presented in table 4. For suspensions containing approximately one million spores per ml. 45 g. of sand for each 25 ml. of suspension produced the most rapid destruction. With glass beads the range of effective action was broader; only when less than 20 g. of beads were used did the number of surviving spores increase materially.

Apart from their greater germicidal efficiency glass beads are superior to the angular abrasives in that they undergo no disintegration with prolonged shaking.

Cell density

Table 5 indicates the extent to which the density of a suspension affected the rate at which spores were killed. In principle this is the converse of the preceding experiment. The percentage of spores killed by five hours' shaking increased slightly but progressively as the concentration of spores was decreased between 100 million and 10 thousand. At about the one thousand level the

TABLE 5

The action of sand when shaken with distilled water suspensions of spores varying in their concentration

CONCENTRATION OF SPORES	VIABLE SPORES AFTER 5 HOURS' SHAKING	
	B. cohaerens	Per cent reduced
per ml.	*per ml.*	
900	51	94.3
10,200	520	95.0
100,000	5,900	94.1
1,040,000	70,000	93.2
10,400,000	956,000	90.8
103,000,000	12,154,000	88.2

germicidal efficacy of the procedure was somewhat less. The prolonged use in shaking procedures of one sample of abrasive apparently does not alter its effectiveness since a sample of sand shaken in water for 170 hours remained unchanged in its action upon spores.

Rate of shaking

Table 6 shows the influence of speed of shaking upon sporicidal efficiency. A speed of 570 s.p.m., which is about the maximum for the apparatus, was most effective, but greater germicidal efficiency might well be obtained with higher speeds.

Relationship between resistance to mechanical destruction and to heat

The term "resistance" as it is used with reference to bacterial spores has come in recent years to have a narrower connotation. Unusual capacity to withstand one destructive agency no longer

TABLE 6

The action of abrasives upon spores suspended in water, as influenced by the speed of shaking

CULTURE	ABRASIVE	VIABLE SPORES BEFORE SHAKING	VIABLE SPORES AFTER 5 HOURS' SHAKING AT			
			330 s.p.m.	430 s.p.m.	530 s.p.m.	570 s.p.m.
		per ml.	*per ml.*	*per ml.*	*per ml.*	*per ml.*
B. cohaerens	Glass beads	1,110,000,	274	21	11	10
B. cohaerens	Sand	1,110,000	270,000	65,000	23,000	11,400
#9499	Glass beads	1,190,000	1,900	92	51	39

s.p.m. = shakes per minute.

TABLE 7

The action of sand when shaken with bacterial spores of differing resistance to heat

ORGANISM	VIABLE SPORES		PER CENT REDUCTION	THERMAL DEATH-TIME AT 115°C.
	0 hours	After 5 hours' shaking		
	per ml.	*per ml.*		
B. cohaerens	1,230,000	74,000	93.9	4 minutes
B. megatherium	840,000	1,730	99.7	5 minutes
#9499	1,320,000	350,000	73.4	15½ minutes
#1518	1,260,000	40,000	96.8	4½ hours

implies a correspondingly high degree of resistance to other lethal influences. That there should exist any correlation between the thermal resistance of spores and their susceptibility to mechanical destruction seemed improbable. An experiment designed to test this point was performed. The results obtained, shown in table 7, are of interest.

Susceptibility to mechanical destruction was measured by the

number of spores[1] which remained viable after 5 hours of shaking with sand. Thermal death-time determinations were made upon similar concentrations of the same cultures suspended in the same medium. The species most resistant to shaking with sand (#9499), was of intermediate heat-resistance; #1518, possessing excessive resistance to heat, was second only to *B. megatherium* in susceptibility to shaking. Spores of *B. megatherium*, though more resistant to heat than those of *B. cohaerens*, proved to be the most susceptible to destruction by abrasives. Although it is recognized that these data are not directly comparable, they

TABLE 8

The action of glass beads when shaken with suspensions of E. coli

SUSPENSION MEDIUM	VIABLE ORGANISMS AFTER SHAKING FOR		SURVIVAL
	0 hours	5 hours	
	per ml.	*per ml.*	*per cent*
Broth	500,000,000*	55,000	0.01
Distilled water	335,000,000†	0.6	0.0000001
Distilled water	3,350,000‡	0	0

* 18 hour extract broth culture.

† Recovered from centrifuged portion of the broth culture, washed twice and resuspended in water.

‡ A dilution of †.

indicate clearly that there is little correlation between the thermal resistance of spores and their resistance to mechanical disintegration.

Effect on vegetative cells

In the final experiment the shaking procedure was applied to a non-sporeforming species. One portion of the cells was shaken in the broth in which they were cultivated, while other portions of the washed cells were shaken for a similar period in sterile distilled water. The results are shown in table 8.

[1] The spores of the different species are not uniform in size, a fact which may have had some bearing upon their relative susceptibility to mechanical destruction.

The effect of broth in reducing the germicidal efficiency of the method is evident. Excessive foaming induced by agitation of the broth acts probably to retard the velocity of the striking particles. If these data are compared with those previously obtained with spores, the greater susceptibility of the vegetative forms becomes apparent. Suspensions of spores always contained some viable cells after five hours' shaking with glass beads, while suspensions containing more than three times as many cells of *E. coli* were rendered sterile by such treatment.

DISCUSSION

The shaking of fluid suspensions of bacteria or their spores with sand or other abrasives is a rather widely used method for dispersing cell aggregates. A danger inherent in this practice is emphasized by the foregoing results. Not only is a killing of the organism to be considered, but also a possible alteration of their functions as a result of injury.

The greater efficiency of the particles spherical in form probably derives from the fact that they apply a greater surface against interposed objects than particles of angular form. Greater germicidal efficiency than that obtained in our study could be achieved by certain modifications in the construction of the shaking apparatus or its accessories. These might include an increase in amplitude of the stroke, increased speed, and sample receptacles held horizontally and designed so as to eliminate pockets, crevices, and other irregularities that afford protection for organisms.

Shaking procedures, as herein described, offer a promising field of usefulness in the study and production of the endoenzymes of microörganisms. H. H. Brown of this laboratory has repeatedly obtained invertase in high concentration from yeast suspensions shaken with sand; this method has proved to be simple, efficient, and much less time-consuming than the autolytic method commonly employed.

The conventional method of preparing bacterial antigens involves a partial denaturation of the protein; it is not unreasonable

to suppose that the immunologic properties of such protein might be inferior to undenatured protein which is obtained when mechanical methods of killing are employed. This hypothesis finds some support in the recent significant papers of Chambers and Flosdorf (1936); Flosdorf, Kimball and Chambers (1939); and Flosdorf and Kimball (1940); in which sonic extracts of *Hemophilus pertussis* in Phase I were found to absorb the agglutinins from homologous rabbit antiserum, a property not possessed by the ordinary commercial preparations. Of interest also is the paper of Mudd and Lackman (1940) who found at least two serologically active components in the neutral sonic extract of group A hemolytic streptococci.

BRIEF SUMMARY

Bacterial spores and vegetative forms are progressively destroyed when their fluid suspensions are agitated long and vigorously with finely divided abrasives. Spores so treated die at a rate which corresponds with the unimolecular law for chemical reactions. The cumulative result of abrasion and repeated collisions between the abrasive particles and the bacteria is a gradual disintegration of the cells. This is attended by a material decrease in opacity of the suspension, a fact which indicates that the cells are reduced to a very fine state of division.

When the shaking is performed at a fixed speed and a constant volume of suspension is employed, the rapidity with which spores are destroyed differs greatly with the nature and quantity of the abrasive used, the size of the particles, and the species and density of the spore suspension. Highest sporicidal efficiency was obtained with small glass beads (60/80). Sporicidal efficiency was progressively increased as the speed of shaking was increased between 330 and 570 r.p.m.

Vegetative forms (*Escherichia coli*) are more rapidly killed by shaking procedures than spores; foam-producing substances reduce somewhat the germicidal efficiency of such procedures.

Resistance of spores to mechanical destruction is apparently not correlated with thermal resistance.

BIBLIOGRAPHY

CHAMBERS, L. A., AND FLOSDORF, E. W. 1936 Sonic extraction of labile bacterial constituents. Proc. Soc. Exptl. Biol. Med., **34**, 631–636.

CURRAN, H. R., AND EVANS, F. R. 1937 The importance of enrichments in the cultivation of bacterial spores previously exposed to lethal agencies. J. Bact., **34**, 179–189.

FLOSDORF, E. W., KIMBALL, A. C., AND CHAMBERS, L. A. 1939 Studies on *H. pertussis*. I. Liberation by sonic vibration of a soluble component that absorbs phase I agglutinins. Proc. Soc. Exptl. Biol. Med., **41**, 122–126.

FLOSDORF, E. W., AND KIMBALL, A. C. 1940 Comparison of various physical means of liberation of the agglutinogen from *H. pertussis* in phase I. J. Immunol., **39**, 287–295.

MELTZER, S. J. 1894 Ueber die fundamentale Bedeutung der Erschütterung für die lebende Materie. Z. Biol., **30**, 464–509.

MELTZER, S. J. 1892 On the importance of vibration to cell life. N. Y. Med. J., **56**, 708–712.

MUDD, STUART, AND LACKMAN, D. B. 1940 The antigenic structure of hemolytic streptococci of Lancefield group A. VI. The immunological behavior of the nucleo-proteins extracted at neutrality. J. Immunol., **39**, 495–509.

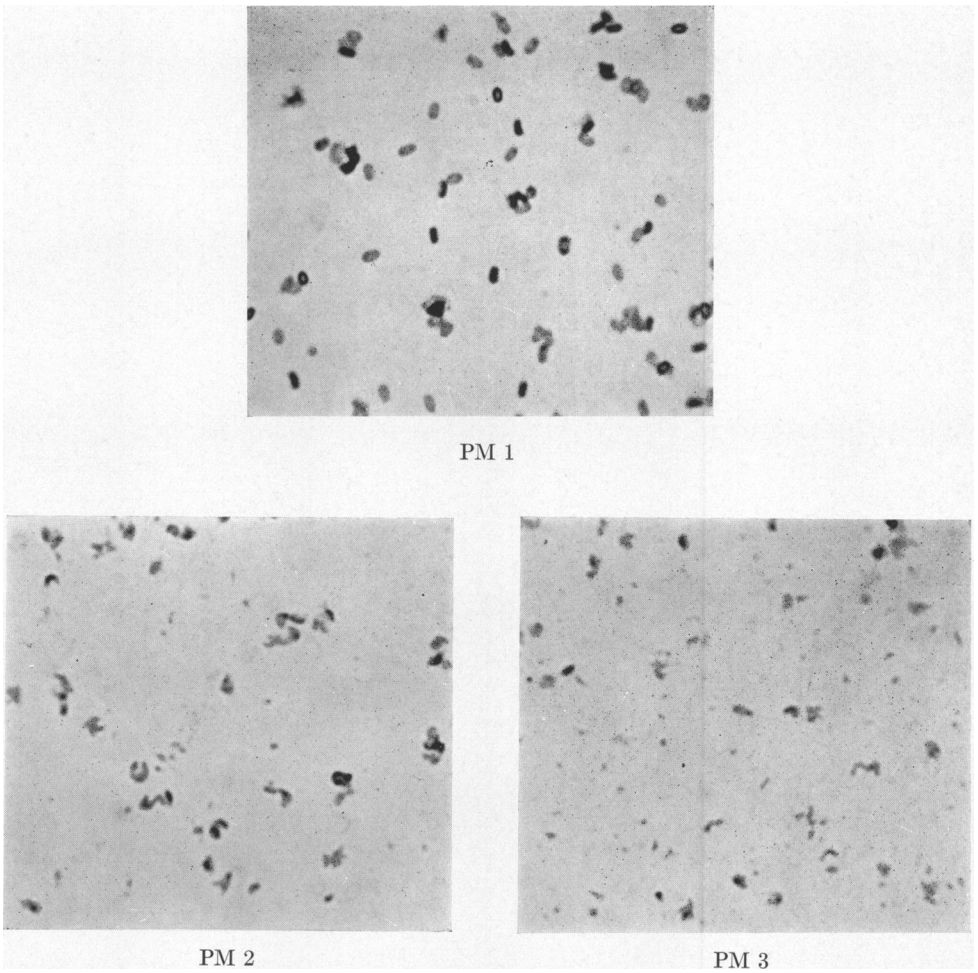
TONE, F. J. 1939 Abrasives 1918–1938. Chem. Industries, **45**, 133–139.

PLATE 1

PM 1.—*B. subtilis* spores before shaking × 1600

PM 2.—*B. subtilis* spores after 5 hours' shaking with glass beads × 1600

PM 3.—*B. subtilis* spores after 22 hours' shaking with glass beads × 1600



PM 1

PM 2

PM 3

(Harold R. Curran and Fred R. Evans: Killing of Bacterial Spores by Agitation)