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KINETICS OF DRY RUPTURE OF BACTERIAL SPORES IN THE PRESENCE OF SALT

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ABSTRACT

SACKS, L. E. (U.S. Department of Agriculture, Albany, Calif.), PETER B. PERCELL, RICHARD S. THOMAS, AND GLEN F. BAILEY. Kinetics of dry rupture of bacterial spores in the presence of salt. J. Bacteriol. **87**:952-960. 1964.—The kinetics of breaking spores in the dry state by use of an excess of sodium chloride and a steel ball in a shaking device were investigated. Under most conditions, disruption is a first-order process. The disruption-rate constant varies directly with the weight of the ball and inversely with the weight of the capsule contents (spores plus salt). Different spore batches differ somewhat in susceptibility to dry rupture. The dry-rupture process is highly reproducible and it is relatively simple to obtain preparations in which exactly 50%, or 90%, of the spores are broken. The procedure is uniquely suited to the disruption of small (5 to 20 mg) samples, but 150 mg of spores have been handled with conventional equipment. Apparently, the chief function of the salt is to separate the spores from one another with a relatively hard, energy-nonabsorbing matrix, preventing aggregation and consequent cushioning of the ball's impact. However, under certain conditions (small ball, high salt, large crystals) appreciable breakage results from collisions of spores with the salt crystals. The minimal salt-spore ratio for efficient breakage depends on the spore batch, but is usually greater than 3:1. Fine glass beads or inorganic salts other than sodium chloride will also serve as the matrix. Electron micrographs of the spores in various stages of disruption are shown, as are electron micrographs of the spore coats of *Bacillus macerans*, *B. megaterium*, *B. cereus*, *B. coagulans*, and *Clostridium bifermentans*. Prolonged agitation disintegrates spore coats. The spore coats of *B. macerans* exhibit a characteristic ribbed structure, previously detected only by carbon replicas of intact spores. Possible application to other biological materials is considered.

The importance of breaking spores to study their enzymatic constitution, spore coats, and biochemical composition is well recognized. A variety of methods have been developed to rupture spores, but all of the commonly used techniques involve violent agitation of spore slurries in the presence of micron-sized glass beads. These methods are subject to such disadvantages as loss of components by adsorption, surface denaturation, frothing, and the inconvenience of removing glass beads. Some of the methods are slow, and some involve expensive equipment.

Recently, Sacks and Bailey (1963) described a method for breaking spores dry in the presence of sodium chloride by shaking them in a Wig-L-Bug dental amalgamator with a steel ball. The method is rapid, efficient, simple, highly reproducible, requires no expensive equipment, and eliminates the need for glass beads with their attendant disadvantages.

The purpose of the present investigation was to determine the optimal conditions for this type of rupture, the maximal amount of spores which can be conveniently broken, some characteristics of the kinetics of the rupture process, and the utility of the technique in the preparation of spore coats.

MATERIALS AND METHODS

Spores. Lyophilized spores of *Bacillus megaterium* NRRL B-938, *B. coagulans* NCA strain 43P, and *Clostridium bifermentans* were prepared as described by Sacks and Alderton (1961). Spores of *B. cereus* strain T were prepared as described by Stewart and Halvorson (1953). *B. macerans* spores were prepared by growing for 3 days at 35 C on a medium patterned after that of Powell and Strange (1956) modified to the following composition: $CaCO_3$, 1%; yeast extract, 1%; Casamino Acids, 1%; agar, 1.5%, in potato extract. The spores were washed, treated with a trypsin-lysozyme mixture to remove sporangial debris (Brown, Ordal, and Halvorson, 1957; Grecz, Annellis, and Schneider, 1962), and purified in a phosphate-polyethylene glycol system (Sacks and Alderton, 1961). Dark spores were

removed by the method of Long and Williams (1958), slightly modified.

Spore rupture. A model 3A Wig-L-Bug dental amalgamator, kept in a refrigerator (5 to 6 C), was employed in all experiments. Stainless-steel vials (⅜ by 1¼ in.) were used in all experiments. In a few cases, specified in the text, ½-in. vials were employed. A single bearing ball of stainless or chrome steel was used in each capsule. These balls were obtained in a variety of sizes, and the ball weight is specified in the appropriate tables. Sodium chloride, analytical reagent, 50 to 100 mesh (through 50, retained by 100 per in. mesh), was used in all experiments unless otherwise stated.

After rupture, spores were taken up in cold, glass-distilled water or 0.1 M tris(hydroxymethyl)-aminomethane buffer (pH 7.6).

Dipicolinic acid (DPA). DPA in spore extracts clarified by centrifugation was estimated from the absorbance at 270 mμ by means of a recording spectrophotometer.

Turbidimetry. Fractured spores were diluted with water to represent 0.1 mg of original spore load per ml. Broken spores were dispersed in water with a vortex mixer, diluted, and transferred to optically matched 18-mm test tubes. Light transmission at 650 mμ was measured in a spectrophotometer.

Electron microscopy. Ruptured spores were washed twice by centrifugation at 11,000 × *g* at 1 C in water, made up to 10 mg/ml (based on original spore concentration), and sprayed on SiO-coated grids. The grids were examined in an electron microscope, operated at 50 kv.

Soluble protein. Soluble protein was determined by the method of Lowry et al. (1951).

Glucose dehydrogenase. Glucose dehydrogenase was determined by the method of Bach and Sadoff (1962).

RESULTS

The rate at which spores are broken in the dry-rupture process was determined by shaking a series of identically loaded steel capsules for varying lengths of time. The capsule contents were dispersed in a known volume of cold water, and the turbidity of the resulting suspension was determined as an index of the extent of breakage. ("Breakage" is defined as a mechanical injury to the spore which results in loss of refractility and DPA.) The rate of breakage indicated by turbidimetric determinations correlated very well

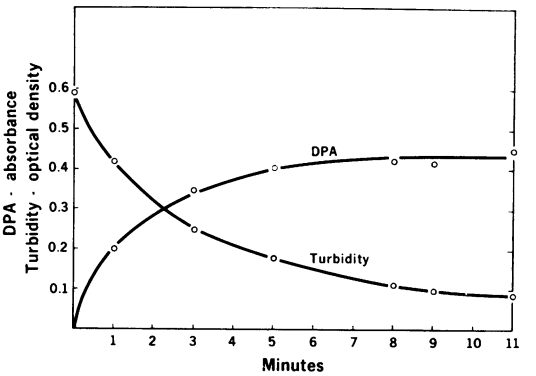

FIG. 1. *Course of spore rupture, as determined by turbidity decrease and DPA release. A 10-mg amount of lyophilized spores of Bacillus megaterium B-938 (lot VIII, 80 HP) was fractured by a 130-mg ball in the presence of 50 mg of NaCl crystals. DPA was measured in extracts representing 0.10 mg of spores per ml in 1-cm cells at 270 mμ.*

with the rate indicated by the release of dipicolinic acid determined spectrophotometrically after centrifugation of the aqueous suspension. [DPA values generally indicated a slightly (5 to 10%) greater degree of breakage than did turbidimetry. This is probably a reflection of the greater opportunities for germination occurring during the centrifugation steps.] The results of a typical experiment are shown in Fig. 1. The spores broken in this experiment were examined by electron microscopy at various stages of fragmentation (see Fig. 2). As breakage approaches completion, some spore coats are severely disintegrated. The spore coats which remain after rupture still exhibit light-scattering properties, which diminish relatively slowly as the coat fragments become smaller and are solubilized. If the turbidity contributed by the spore coats (optical density ≃0.1) is subtracted from the total turbidity, the resulting figure is proportional to the number of unbroken spores. (Optical density was estimated from a series of turbidimetric determinations at various time intervals after 99% rupture, and extrapolation of these points back to time zero.) A plot of the log per cent unbroken spores gives a straight line (Fig. 3), indicating that this type of breakage is a first-order process (*see* Curran and Evans, 1942; Rahn, 1945). In this, and all similar experiments, the slope was calculated from the experimental points by the method of least squares. The slope of this straight line is designated as the rupture rate constant, *R*.

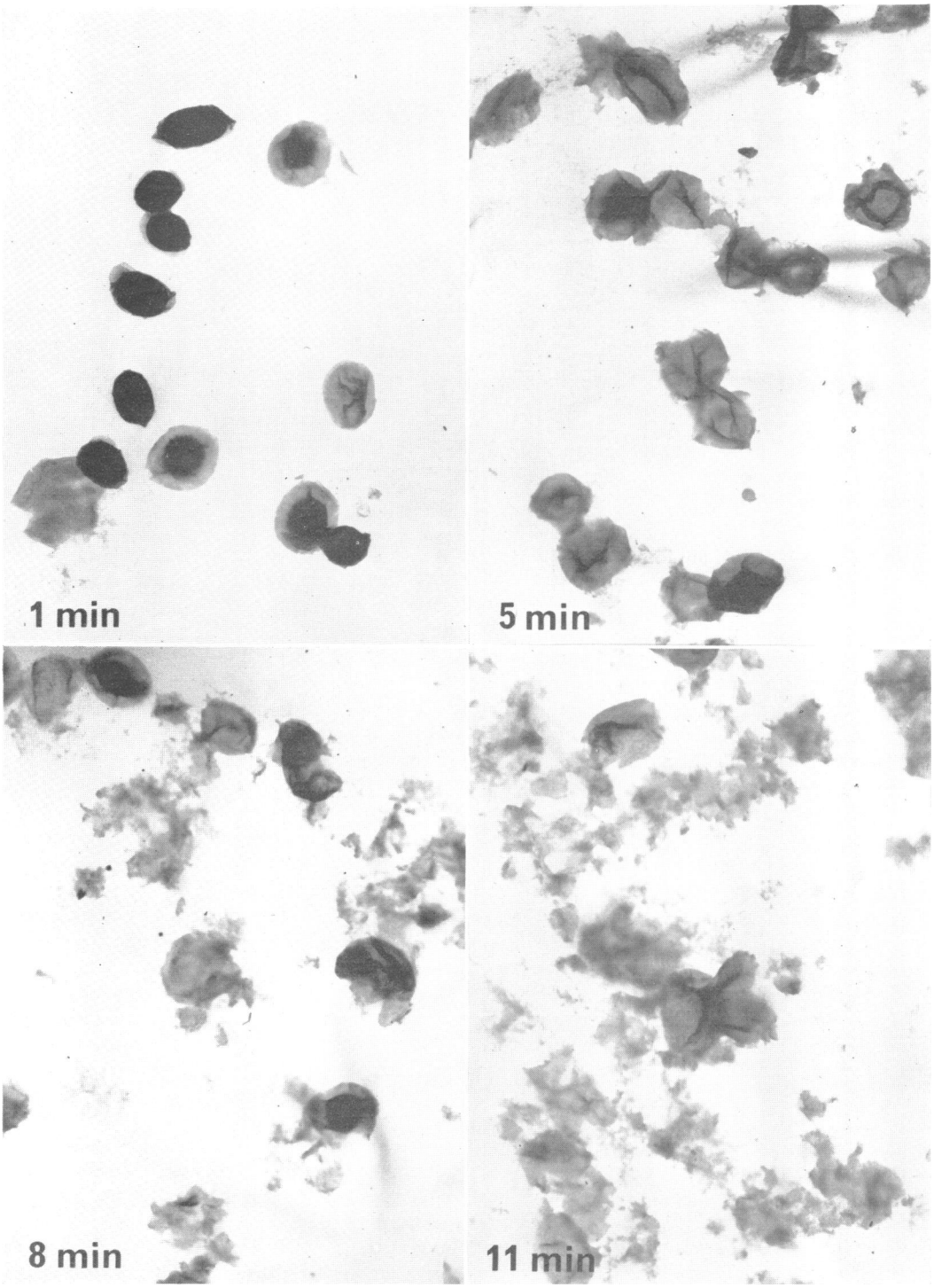

FIG. 2. *Electron micrographs of Bacillus megaterium spores in various stages of disruption. These micrographs were made in the experiment described in Fig. 1. Time of breakage is indicated. Note that in the early stages of breakage there are a number of enlarged forms, with an electron-dense core, which have survived two water washes.* × *6,600.*

A series of experiments, in which R was determined when various weights of spores and salt were used with balls of different weights, showed that the rupture rate varies directly with the weight of the ball, and inversely with the weight of the capsule contents (see Table 1; Fig. 4). [Each batch of spores exhibits a characteristic susceptibility to dry rupture. *B. cereus* lot 12/8 and *B. megaterium* lot VIII, 80 showed R values of -0.18 and -0.10 when broken under the same conditions (130-mg ball, 120 mg of salt, 10 mg of spores).]

This relation holds for nearly all circumstances. Only if there is insufficient salt relative to spores or if a small ball is used in conjunction with a large amount of salt do the results deviate from the relationship formulated above.

Figure 5 shows the results of an experiment which was designed to demonstrate both types of exception, acting simultaneously. In this experiment, varying amounts of NaCl were added to a series of capsules, each containing 5 mg of *B. megaterium* spores and a small (55 mg) ball. Each capsule was shaken for a time which should have produced 50% breakage, as predicted from the data of Fig. 4. Under the chosen conditions, the theoretical breakage was achieved only when 40 or 70 mg of salt were employed (depending on whether DPA release or turbidity is used as an index of breakage). This represents a salt-spore ratio of somewhere between 8:1 and 14:1.

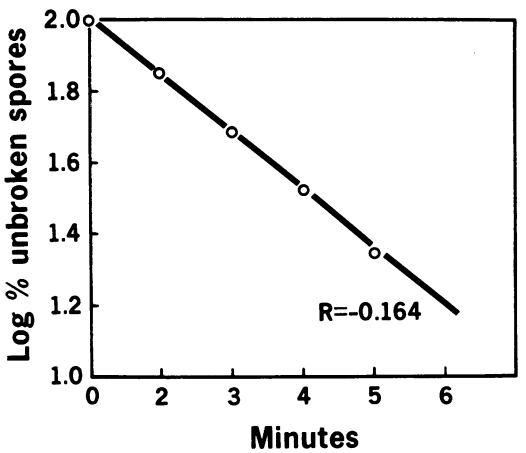

FIG. 3. *Course of spore rupture, plotted as log per cent unbroken spores vs. time. Capsule contents: 10 mg of lyophilized spores of Bacillus megaterium NRRL B-938 (lot 8/62 GA) and 90 mg of NaCl; ball weight, 250 mg. Rupture rate, R, determined by method of least squares.*

TABLE 1. *Influence of capsule contents on rupture rate*

Amt of spores*	Amt of NaCl	Wt of ball	No. of determinations†	Ball / Salt + spore	$-R$
mg	*mg*	*mg*			
10	101.0	55.4	4	0.5	.035
10	120.0	130.1	4	1.0	.093
10	76.7	130.1	4	1.5	.085
30	113.0	250.8	3	1.75	.113
10	115.0	250.8	4	2.05	.167
10	90.2	250.8	4	2.5	.165
10	73.6	250.8	4	3.0	.176
5	343.0	1,043.0	4	3.0	.220
10	61.0	250.8	7	3.5	.214
5	57.7	250.8	3	4.0	.283

* Lyophilized spores of *Bacillus megaterium* NRRL B-938, lot 8/62 GA.

† Number of experimental points at various times, to determine R.

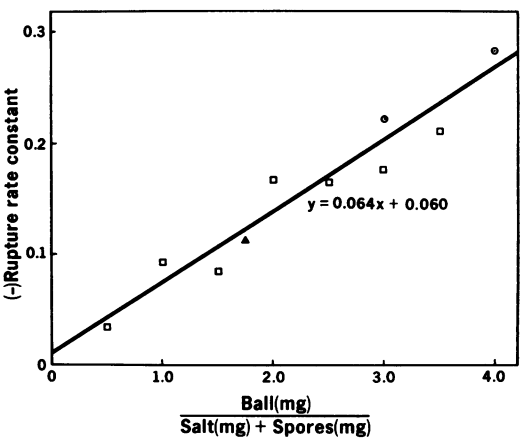

FIG. 4. *Influence of ball size and capsule contents on rupture rate of spores of Bacillus megaterium NRRL B-938 (lot 8/62 GA). See Table 1 for experimental details. Symbols: ⊙, 5 mg of spores; □, 10 mg of spores; ▲, 30 mg of spores. Slope calculated by method of least squares.*

The spores in the capsules which contained little or no salt were often highly aggregated, and difficult to disperse after breakage. It appears that a major function of the salt is to separate the spores from one another with a hard, energy-nonabsorbing matrix, preventing them from aggregating with consequent cushioning of the interior spores from the ball's impact. Figure 5 also shows that very large amounts of salt result

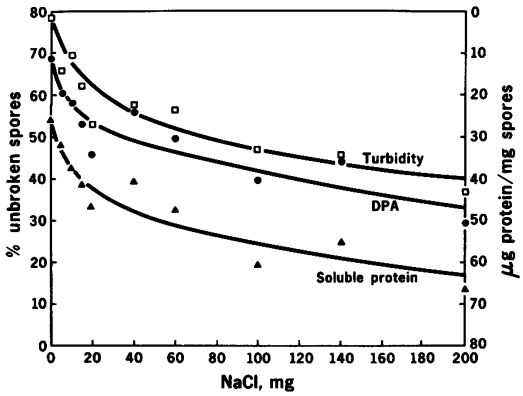

FIG. 5. *Influence of NaCl level on rate of spore rupture. All capsules contained 5.0 mg of Bacillus megaterium spores and a 55.4-mg ball, in addition to the specified amount of NaCl. Each capsule was shaken for a time sufficient to rupture 50% of the spores, as determined from the data of Fig. 4. Turbidimetric and DPA analyses plotted as per cent unbroken spores.*

in a somewhat greater degree of breakage than might be predicted. This is due to the fact that NaCl crystals of 50 to 100 mesh have some disruptive capacity, even in the absence of a ball.

When a large salt-spore ratio was employed with a small ball, the disruptive effect of the salt became very evident. Figure 6 shows the results of an experiment in which a small ball was used in conjunction with several salt-spore ratios. Even though the capsule contents were designed to yield identical rates, the *initial* rates differ widely, being greatest where the salt-spore ratio is highest. However, as the salt particles are ground down (Kramm and Stone, 1963), their disruptive capacity lessens, and all the curves become parallel, at approximately the calculated disruption rate.

In the experiment shown in Fig. 7, the steel ball was omitted to illustrate the disruptive effects of large salt particles. Curves B and C contrast the rupture rates of prepulverized and normal-sized (50 to 100 mesh) salt, and show that the larger salt crystals have a much higher rupture rate. Curves A and C show that larger amounts of salt result in much higher rupture rates.

Under the conditions of most of our experiments, breakage due to the action of salt alone is usually negligible since the rupture rate, in the presence of a reasonably heavy ball, is relatively high and the salt is rapidly pulverized. Under such circumstances, the initial size of the salt crystals will not affect the rupture rate. An experiment in which 10 mg of *B. megaterium* spores (GA 8/62) were ruptured by a 130-mg steel ball in the presence of 76 mg of either 50- to 100-mesh crystals or prepulverized salt gave identical values for R (-0.085).

The influence of substituting other materials for NaCl is shown in Table 2. It seems probable that the increased effectiveness of NaCl over KBr is due to its greater hardness. However, the much greater efficiency of glass may be due partially to its greater hardness, but mostly to the existence of irregular, jagged pieces which result from crushing of the beads by the steel ball (*see* Rode and Foster, 1960).

The effect of prolonged agitation on protein and glucose dehydrogenase release is shown in Fig. 8. This experiment indicates that an optimal disruption period exists for maximal recovery of active enzyme. One possible explanation for decreased yields of glucose dehydrogenase on

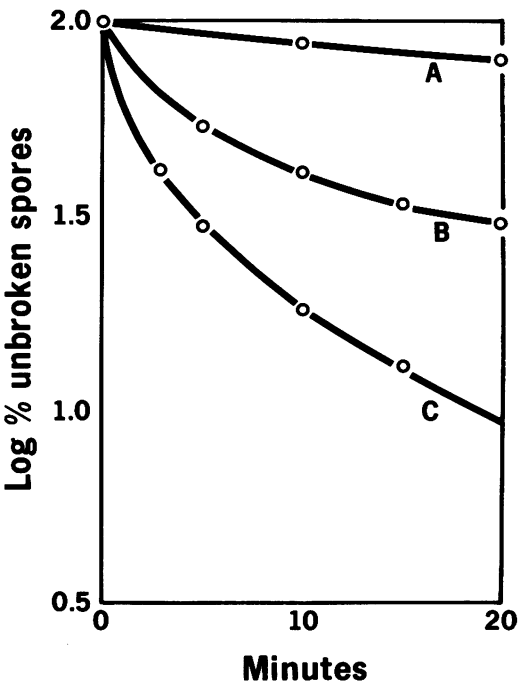

FIG. 6. *Disruptive effect of salt crystals on Bacillus megaterium spores. Salt-spore ratios: (A) 8.23; 30 mg of spores, 247 mg of salt; (B) 26.7; 10 mg of spores, 267 mg of salt; (C) 54.4; 5 mg of spores, 272 mg of salt. NaCl crystals, 50 to 100 mesh; 55.4-g ball.*

prolonged rupture is denaturation due to heating. A thermocouple attached to the capsule indicated that the temperature rose as much as 7.5 C (to about 13 C) in prolonged runs. Furthermore, if the capsule is allowed to stand in place after completion of a run, the heat from the idle motor may raise the temperature another 5 C. Further evidence that heat was partially responsible for decreased enzyme yields was obtained from experiments conducted at −40 C, which showed that yields of active enzyme after 20 min of agitation were approximately twice the yields obtained in comparable experiments at 6 C. Mechanical damage to the enzyme seems to be equally important, however, since even at −30 C peak recoveries were obtained before the spores were completely broken.

Some attempts have been made to increase the amount of spores which can be broken with a model 3A Wig-L-Bug. With a 6:1 salt-spore mixture and a 250-mg ball, it was possible to rupture 80 mg of *B. cereus* spores in a ⅜-in. capsule in about 12 min. This figure probably approaches the maximum that can be handled in this size vial, which is very nearly full when a fluffy spore preparation is used. It was, however, possible to rupture 150 mg of spores in 6 min with a 1,035-mg ball by using a ½ by 1 in. vial.

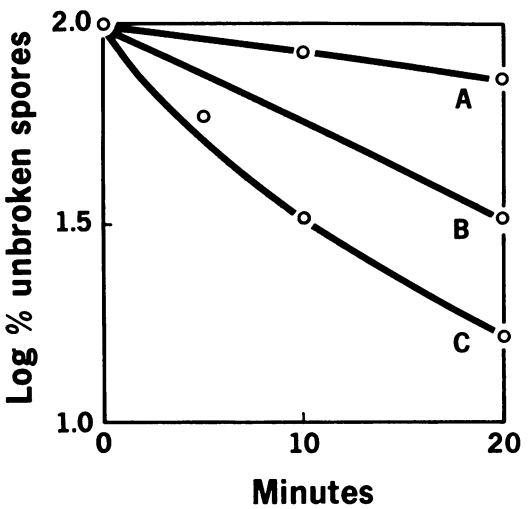

FIG. 7. *Influence of crystal size and amount of NaCl on rupture of spores in the absence of a steel ball. Capsule contents: (A) 5 mg of Bacillus megaterium spores, 25 mg of NaCl (50 to 100 mesh); (B) 5 mg of spores, 250 mg of NaCl (prepulverized for 5 min with a 55-mg ball); (C) 5 mg of spores, 250 mg of NaCl (50 to 100 mesh).*

TABLE 2. *Influence of inorganic matrix on rupture rate*

Inorganic matrix (description)	Rupture rate		Hardness (Moh's scale)‡
	No ball*	250.8-mg ball†	
KBr (analytical reagent, infrared grade)		0.1301	≤2
NaCl (analytical reagent, 50 to 100 mesh)	0.0395	0.1645	2
Glass (Minnesota Mining, Superbrite)	0.0404	0.4915	6.0

* A 5-mg amount of *Bacillus megaterium* spores (GA 8/62) plus 250 mg of matrix.

† A 10-mg amount of *Bacillus megaterium* spores (GA 8/62) plus 90.2 mg of matrix.

‡ Hodgman, Weast, and Selby (1958).

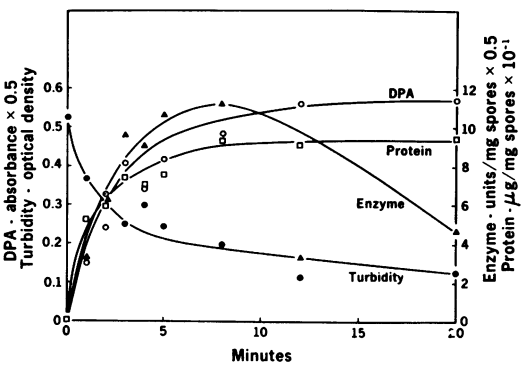

FIG. 8. *Influence of shaking time on recovery of active enzyme. Contents of each capsule: 10 mg of spores of lyophilized Bacillus cereus T, 120 mg of salt, and a 130-mg ball. DPA was measured in extracts representing 0.33 mg of spores per ml in a 1-cm path.*

Some spore coats of various species were prepared by stopping the rupture process well before all of the spores were broken, and washing away the salt by two centrifugations in cold water. These preparations were then sprayed onto grids and examined by electron microscopy (Fig. 9). The coats of *B. macerans* (Fig. 9A) are particularly striking. The ribbed nature of the coat of this organism had been demonstrated previously only by carbon replicas (Franklin and Bradley, 1957). Also noteworthy is the fact that the exosporium still surrounds the empty coats of *B. cereus* (Fig. 9B).

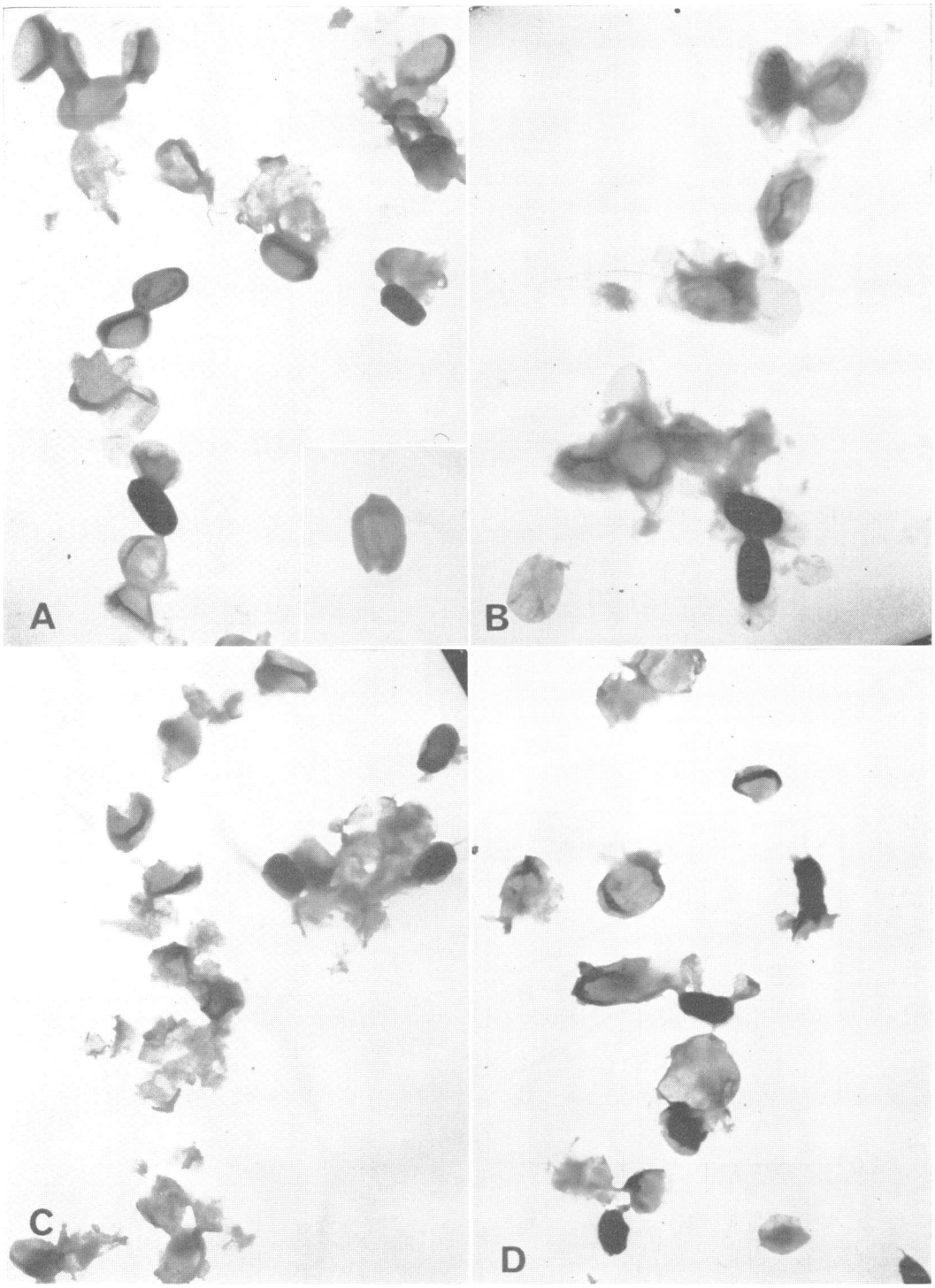

FIG. 9. *Spore coats of various species obtained by dry rupture. A 10-mg amount of lyophilized spores was broken by a 130-mg ball in the presence of 30 to 60 mg of NaCl for 3 min. Shaking was stopped well before the spores were completely ruptured, to avoid extensive pulverization. For this reason, numerous intact spores are evident.* (A) *Bacillus macerans (note ribbed structure).* (B) *B. cereus T (note exosporia still attached).* (C) *B. coagulans.* (D) *Clostridium bifermentans.* × *6,600.*

DISCUSSION

Dry rupturing of spores with salt is a versatile technique which permits considerable flexibility in the treatment of the broken spores. Depending on the particular requirements of the experiment, the broken spores may be handled in one of three ways. (i) They may be treated with a polar solvent, dissolving away the salt, and retaining only particulate matter insoluble in the solvent. (ii) They may be treated with a nonpolar solvent. Presumably, this would result in the retention of some different spore constituents as insoluble particulate matter. The salt might be removed by some physical technique (e.g., density-gradient centrifugation). (iii) Solvent treatment may be omitted. It is conceivable that certain types of investigation (e.g., electron microscopy of carbon replicas) might be fruitful with such a preparation. The nonaqueous methods are of particular interest, in view of the possibility that DPA exists in the spore as a water-labile constituent (Foster, 1959).

Dry rupturing of spores with salt is a highly reproducible process, and it is relatively simple to obtain preparations in which 50%, or 90%, of the spores are "broken." This property may be especially valuable in certain types of studies, for example, investigations of spore coats, where the degree of disintegration must be controlled.

The method described is particularly well adapted to the handling of small samples (e.g., 5 mg) and quantitative recovery of the released components (e.g., DPA). This suggests the utility of dry rupture in various microanalytical investigations. The method is not confined to small samples, however.

Although the model 3A Wig-L-Bug probably cannot be employed for spore samples of more than 300 mg, it seems entirely possible that the dry-rupture method could be satisfactorily employed with almost any type of device to provide rapid agitation. No claim is made that the Wig-L-Bug is the only suitable device. The Wig-L-Bug capsule describes a figure eight motion about 2.1 cm long by 0.35 cm wide, and is stated by the manufacturer to operate at 3,150 to 3,250 rev/min. The length of the motion is slightly sensitive to capsule gross weight. The model 3A used here is not designed for heavy loads or sustained operation. A heavy-duty model is available and may be satisfactory for large samples. The Nossal disintegrator, equipped with liquid CO_2 jet cooling assembly (Sierra, 1963), may also be satisfactory.

The rupture rate, R, is probably a measure of the rate of "mechanical germination" (Rode and Foster, 1960). Undoubtedly, the turbidity drop and DPA release represent the events which normally follow a mechanical injury to the spore. They do not necessarily imply "disruption" or even death. As a matter of fact, the swollen forms with electron-dense centers seen in an electron microscope may be "mechanically germinated" spores which have suffered no gross fissures. The relative insolubility of the spore core is striking.

The dry-rupture method described here may offer some special advantages in investigations on spore coats. The ease with which coats in various stages of disintegration may be prepared has already been mentioned. Also, very small quantities of spores may be broken and examined with an electron microscope, suggesting utility in taxonomic studies. For example, the ridged coat of *B. macerans* is distinctive, and this ridging can easily be detected by examination of the empty coat, whereas preparation of carbon replicas is a somewhat more complex operation.

Dry rupturing with salt may have considerable application with biological materials other than spores. Preliminary experiments indicate that dried yeast cells, notoriously difficult to break, are easily broken by this technique.

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