GERMINATION OF BACTERIAL SPORES WITH ALKYL PRIMARY AMINES[1]

L. J. RODE AND J. W. FOSTER

Department of Bacteriology, The University of Texas, Austin, Texas

Received for publication November 17, 1960

The germinative action of L-alanine, inosine, and related analogues are designated as "physiological germination" (Rode and Foster, 1960*a*) on the assumption that these substances may play a role in germination in nature. Similar actions of nonmetabolite chemicals on spores are designated as "chemical germination" (Rode and Foster, 1960*a*). Included in this category are ethylenediaminetetraacetic acid, calcium dipicolinate, hydrogen peroxide, and a variety of cationic and anionic surfactants (Brown, 1957; Riemann and Ordal, 1960; Falcone, Salvatore, and Covelli, 1959; Rode and Foster, 1960*b*, *c*). Changes in spores of *Bacillus megaterium* which are induced alike by surfactants and metabolites are: loss in refractility, acquired stainability with crystal violet, enlargement, lessening of optical density of suspensions of spores, loss of about one-third of spore weight, and a discharge of spore dipicolinic acid, mucopeptide, and calcium. However, spores treated with surfactants were unable to form colonies in an agar medium (Rode and Foster, 1960*c*). This situation, in which the changes characteristic of physiological germination are brought about by an agent that also is responsible for the nonviability of the spore, we have named "lethal germination." This term would also be a useful one to describe situations in which spores undergo changes typical of germination under conditions where further vegetative growth is precluded.

Selected members of one class of surfactants, the long chain alkyl primary amines, have been found to bring about the above listed changes in spores. They are also nonlethal. The viable spores thus changed are not heat resistant. These changes, which may be evoked even more rapidly by alkyl amines than by physiological germinants, are indistinguishable from those taking place during physiological germination.

[1] Supported in part by grants from the Microbiology Branch, Office of Naval Research, and the National Institutes of Health. A preliminary note on this subject has been published in Nature, **188**, 1132 (1960).

MATERIALS AND METHODS

Culture procedures. The methods employed with *B. megaterium* have been described previously (Rode and Foster, 1960*a*). Final suspensions of spores in sterile, deionized water consisted entirely of refractile spores which remained unchanged for months during storage at 4 C.

Optical density measurements. A lowering of the optical density of a suspension of spores was used as a presumptive criterion of germination (Powell, 1950). Spore suspensions giving an initial reading of about 120 (10^7 spores per ml) in the Klett-Summerson photoelectric colorimeter (green filter no. 54) were regularly employed. In the text and figures, all values for optical density are expressed as percentage of the initial reading measured in Klett-Summerson photometer units. Routinely, the spores were suspended in 0.066 M phosphate buffer (pH 7.0 to 8.0), the alkyl amine added, and the suspensions incubated in Klett tubes in a water bath at 40 C.

Heat treatments. Prompt physiological germination of the spores of *B. megaterium* is dependent on a preheating (Evans and Curran, 1943) which was standardized at 1 hr at 60 C. Sensitivity to heat as a criterion of the germinated versus the ungerminated state was determined by exposing spores in 0.066 M phosphate buffer (pH 7.0) to 65 C for 20 min followed by quantitative plating on complete medium.

Chemical procedures. Dipicolinic acid analyses were performed by the ultraviolet spectrophotometric method described elsewhere (Perry and Foster, 1955; Martin and Foster, 1958). Its sensitivity was advantageous with the dilute spore suspensions employed routinely in this work. Other analyses made were for protein (Lowry et al., 1951), amino acids (Moore and Stein, 1948), hexosamine (Elson and Morgan, 1933; Immers and Vasseur, 1950), and calcium (Roe and Kahn, 1929). Hydrolysis prior to the hexosamine and amino acid determinations was achieved by heating at 106 C for 3 hr in 6 N HCl.

TABLE 1

*Action of some primary n-alkyl amines on spores of Bacillus megaterium**

n-Alkyl Amine	Minimal Effective Concn	Optical Density of Suspension	Refractility†	Stainability‡
None		No reduction	Normal	−
Heptyl	2×10^{-2} M	Marked reduction	None	+
Octyl	10^{-2} M	Marked reduction	None	+
Decyl	10^{-3} M	Marked reduction	None	+
Dodecyl	10^{-4} M	Marked reduction	None	+
Tetradecyl	10^{-5} M	Marked reduction	None	+
Oleyl	10^{-5} M	Marked reduction	None	+

* Suspension in 0.066 M phosphate buffer (pH 7.2); temperature, 37 C.

† Dark contrast phase illumination.

‡ Fixed smears stained 2 min with gentian violet.

RESULTS

Carbon chain length. Indicative of the germinative properties is the striking change in the optical density of a spore suspension, and in the refractility and stainability of the individual spores. Alkyl amines having a chain length of seven or more carbon atoms are active in these respects (Table 1). The potency increases with chain length; the water insolubility of the longer chain alkyl amines made it difficult to arrive at exact values for their effective concentrations. From the relation between efficacy and chain length, it appears that the surface active properties are responsible for the observed changes. Preheating the spores was not essential. No other organic substances were added in these experiments. *n*-Dodecylamine $CH_3(CH_2)_{10}CH_2NH_2$ was selected for a more detailed study.

Concentration. Fig. 1 shows the effect of the concentration of *n*-dodecylamine on the optical density of a spore suspension as a function of time. Microscopically, these treated spores in the most effective concentrations were nonrefractile and they were stainable. Spores in untreated control suspensions were refractile and unstainable.

Temperatures. Temperature has a marked effect on the change in optical density induced by *n*-dodecylamine. At 60 and 72 C the rate of change was greater and was practically completed in 1 to 2 min, but the degree of clearing finally attained was somewhat less than that attained at 37, 43, and 50 C. The physiological germinants and the chemical germinants can be differentiated by their efficacies at an elevated temperature (Fig. 2; also Halvorson, 1959).

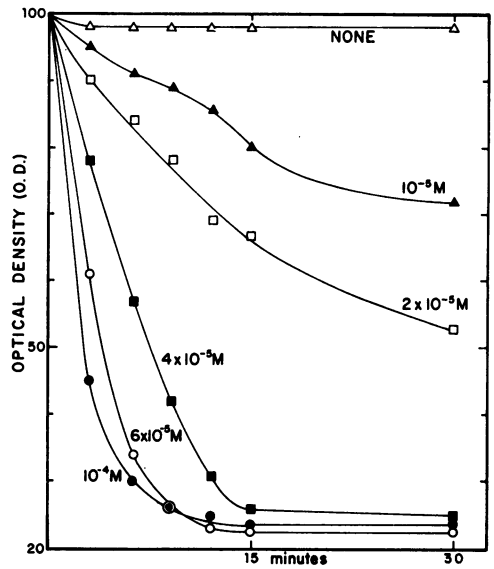

Fig. 1. Reduction in optical density of a suspension of spores of *Bacillus megaterium* due to various concentrations of *n*-dodecylamine. Temperature, 40 C; 0.066 M phosphate buffer, pH 8.0.

pH. The change in optical density induced by *n*-dodecylamine is dependent on the pH of the suspending liquid. As with other cationic surfactants (Rode and Foster, 1960*c*), clearing took place only above a critical pH value, in this case 6.0, and maximally at pH 7.4 to 7.8. The initial rate of clearing at pH 9.2 started out maximally, but the final level was not as great as at pH 7.4 to 7.8. The clearing by *n*-dodecylamine can be stopped abruptly by acidification or by lowering the temperature to 0 C. Restoring

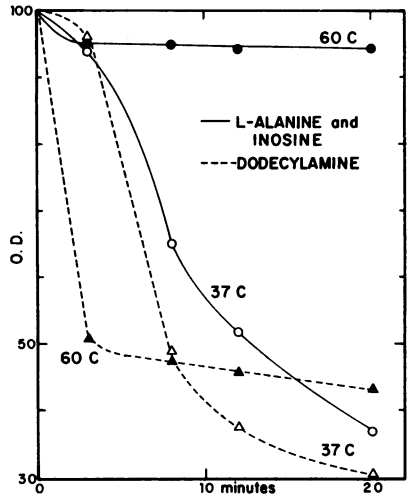

Fig. 2. Optical density changes at two temperatures for spores of *Bacillus megaterium* caused by *n*-dodecylamine (10^{-4} M) and by a mixture of L-alanine (10^{-4} M) and inosine (1.4×10^{-4} M). Preheated spores were used; 0.066 M phosphate buffer, pH 7.0.

the temperature to 37 C also restored the rate of clearing to normal.

Other species. Spore suspensions of *Bacillus cereus*, *Bacillus subtilis*, *Bacillus subtilis* var. *globigii*, and *Bacillus stearothermophilus* were tested with *n*-dodecylamine at 40 and 60 C in the same manner as described for *B. megaterium*. A definite change in optical density was noted in each of the treated suspensions, and it is presumed they are affected alike.

Interference with L-alanine-inosine function by alkyl amines. A common site of adsorption or action was considered in connection with the several changes in spores induced alike by the physiological germinants and by alkyl amines. A difference was found in the abilities of various alkyl amines to block physiological germination. At concentrations too low to cause any change in optical density *n*-heptylamine, *n*-octylamine, and *n*-decylamine inhibited the optical density changes caused by L-alanine and inosine. On the other hand, *n*-dodecylamine, *n*-tetradecylamine, and *n*-oleylamine did not block the physiological germinants. Table 2 illustrates the effects of *n*-decylamine and *n*-dodecylamine. Apart from the similarity of the changes in the spores induced by the physiological germinants and by the alkyl amines, the lack of interference

TABLE 2

*Effect of n-decylamine and n-dodecylamine on optical density changes caused by L-alanine and inosine in a suspension of spores of Bacillus megaterium**

Concn of Alkyl Amine	L-Alanine and Inosine†		Difference
	Absent	Present	
n-Decylamine:			
None	100	34	−66
10^{-4} M	94	94	0
2×10^{-4} M	86	85	−1
4×10^{-4} M	48	44	−4
6×10^{-4} M	25	26	+1
8×10^{-4} M	25	24	−1
10^{-3} M	27	25	−2
n-Dodecylamine:			
None	100	30	−70
10^{-5} M	94	35	−59
2×10^{-5} M	88	31	−57
4×10^{-5} M	72	26	−46
6×10^{-5} M	45	25	−20
8×10^{-5} M	27	24	−3
10^{-4} M	26	24	−2

* Figures represent Klett-Summerson units; time, 40 min; temperature, 37 C.

† L-Alanine, 10^{-4} M; inosine, 1.4×10^{-4} M.

of *n*-dodecylamine with the action of the first named germinants implies that the sites of action may not be unrelated.

n-Dodecylamine and boiled spores. Spores of *B. megaterium* held in buffer at 100 C for several minutes lose practically all of their dipicolinic acid, but their refractility is not reduced to any great extent nor are they readily stainable (Rode and Foster, 1960*b*). Such spores offer an opportunity to test the relation between refractility and surfactants. However, *n*-dodecylamine did not significantly lower the optical density of spores previously exposed to boiling temperature.

Neutralization of n-dodecylamine activity. Certain substances combine with and neutralize the activity of cationic surfactants (Lawrence, 1950), including sodium lauryl sulfate (Fig. 3) and cephalin. These neutralizing substances, at their optimal levels, 40 and 78 μg per ml, respectively, did not reduce the optical density by themselves. Sudden termination of the action of *n*-dodecylamine on bacterial spores is a valuable tool for physiological studies.

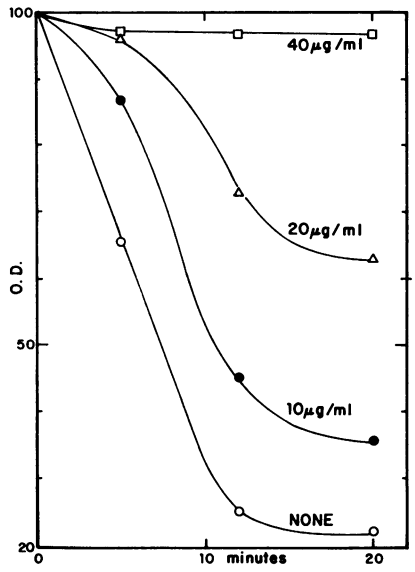

Fig. 3. Neutralization of the action of *n*-dodecylamine on spores of *Bacillus megaterium* by various concentrations of sodium lauryl sulfate. Each treatment had 6×10^{-5} M *n*-dodecylamine in 0.066 M phosphate buffer, pH 8.0; temperature, 42 C.

Following a short exposure of spores of *B. megaterium* to 6×10^{-5} M *n*-dodecylamine in phosphate buffer (pH 8.0), at 42 C, a significant fraction of the spores were viable. This was judged by the formation of colonies when suitable dilutions were spread on the surface of nutrient agar. As seen later, dipicolinic acid is released during this treatment. These spores had lost their heat resistance at 60 C for 30 min. It is probable that the presence of the alkyl amine during the heating contributed to the death of the spores. A concentration of *n*-dodecylamine (6×10^{-6} M) too low to effect a reduction in optical density, dipicolinic acid release, or loss of cell viability, failed to convert resistant spores to heat sensitive spores. Apparently these changes go hand in hand, provided a sufficient amount of the alkyl amine is adsorbed on the spores. Likewise, a concentration of *n*-dodecylamine (6×10^{-5} M) that was very effective in reducing optical density at 40 C was ineffective at 0 C.

Microscopic aspects of spores exposed to n-dodecylamine. Spores of *B. megaterium* suspended in buffer did not stain with gentian violet (Fig. 4*A*), whereas spores treated with *n*-dodecylamine (Fig. 4*C*) stained readily and were no different in this respect from spores germinated with a mixture of L-alanine and inosine (Fig. 4*B*).

Electron microscopy. Unchanged normal spores appeared almost entirely opaque to electrons, with only slight evidence of the outer spore coat in some cases (Fig. 4*D*). An exosporium is lacking in this species. By contrast, the L-alanine-inosine spores (Fig. 4*E*) and the *n*-dodecylamine spores (Fig. 4*F*) show an opaque central core which is somewhat smaller than the unchanged spores, and spore coat(s) that are readily discernible and perhaps somewhat expanded. These two types of spores are essentially indistinguishable under the electron microscope.

Dark contrast phase microscopy. Time lapse microscopy of spores of *B. megaterium* during germination with L-alanine-inosine (Fig. 4*G–J*) and during exposure to *n*-dodecylamine (Fig. 4*K–N*) show in both cases a characteristic loss in refractility.

Physiological changes induced by n-dodecylamine. The parallelism between the effect of *n*-dodecylamine and the germinative changes induced by L-alanine and inosine suggested that *n*-dodecylamine may indeed induce germination. This was established in the following experiment. Samples of a suspension of spores of *B. megaterium* were exposed to *n*-dodecylamine at 40 C for various times, at which they were analyzed as follows: (i) The optical density was measured. (ii) Residual *n*-dodecylamine was neutralized by diluting with a solution of cephalin; samples of suitable dilutions were spread before and after pasteurization at 65 C for 20 min on the surface of nutrient agar plates and the colonies counted after incubation for 24 hr. (iii) A portion of the suspension was filtered rapidly through a membrane filter. This required only 2 to 3 sec. The clear filtrate was analyzed for dipicolinic acid.

Data from this experiment are given in Table 3. For 3 min all of the spores exposed to *n*-dodecylamine (total survivors) remained viable. Thereafter, they were killed rapidly. During the first 3 min, over 97% of the spores were converted to heat-sensitive cells. Concomitantly, although at lower rates, dipicolinic acid was released from the spores and the characteristic reduction in optical density of the suspension took place.

After 1 min, at which time 94% of the population had lost heat resistance, only 18% of the

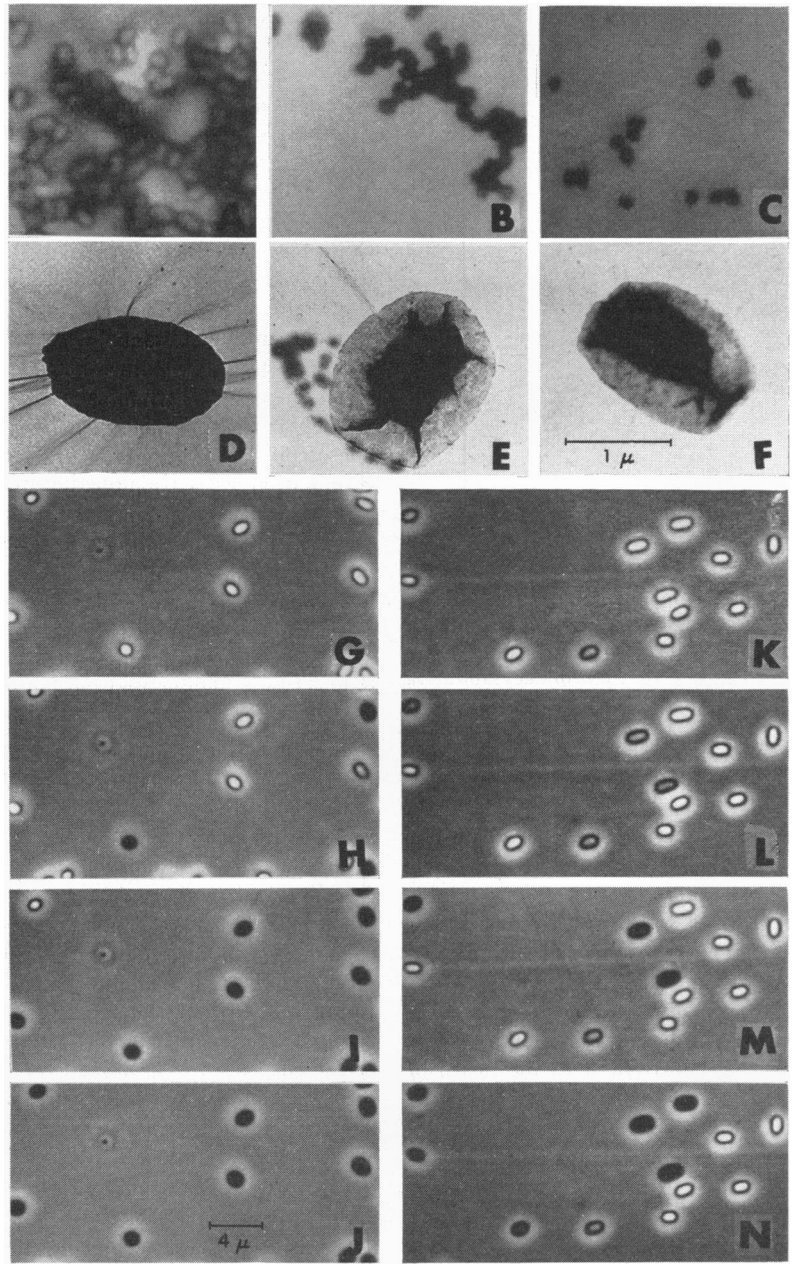

Fig. 4. Microscopic characteristics of spores germinated with *n*-dodecylamine. *A*, *B*, and *C* stained with gentian violet. *A:* ungerminated resistant spores, unstainable. *B:* spores germinated with L-alanine-inosine mixture, stainable. *C:* spores exposed to *n*-dodecylamine, stainable. *D*, *E*, and *F:* electromicrographs of spores, respectively, ungerminated, germinated with L-alanine-inosine mixture, and exposed to *n*-dodecylamine. *G to J:* time lapse series with dark contrast phase microscopy of the same field of spores germinating in L-alanine-inosine mixture. *G*, less than 5 min; *H*, 30 min; *I*, 60 min; *J*, 75 min. Progressive loss of refractility and a darkening of the spores is apparent. *K to N:* time lapse series with dark contrast phase microscopy of the same field of spores exposed to *n*-dodecylamine. *K*, less than 5 min; *L*, 10 min; *M*, 20 min; *N*, 30 min. The progressive loss of refractility and darkening of the spores is similar to that in the previous series representing physiologically germinated spores.

TABLE 3

*Changes in a suspension of spores of Bacillus megaterium exposed to n-dodecylamine**

Time of Exposure to 6 × 10^{-5} M *n*-Dodecylamine	% of Original Spore Population Still Viable	% of Original Spore Population Resistant to Heat†	Optical Density, % of Initial Value	Dipicolinic Acid Release, % of Total
min				
0‡	100	105	100	0
0.5	107	89	100	5.6
1	112	6	94	18
3	111	2.5	61	70
4	86	<1	44	77
5	47	<1	38	80
6	29	<1	33	82
8	14	<1	29	87
10	3.5	<1	25	94

* Spores suspended in 0.066 M phosphate buffer (pH 8.0); temperature, 40 C.

† These cells survived heating for 20 min at 65 C.

‡ No *n*-dodecylamine in this tube.

dipicolinic acid had been released into the suspending liquid. It was possible to demonstrate that the retained dipicolinic acid was diffusible from the spores, given enough time. For example, in a separate experiment the *n*-dodecylamine was neutralized with cephalin or by high dilution. The loss of dipicolinic acid from the spores continued until these cells were devoid of the compound. Such experiments indicate that the *n*-dodecylamine, once its action on the bacterial spore has been initiated, is not essential for the subsequent release process. The secondary release was markedly retarded by a low pH or by a low temperature; at room temperature the release proceeded quite rapidly. In none of the following controls were any of the changes described in Table 3 observed during a 10-min period; a suspension in *n*-dodecylamine solution at 0 C; the same at pH 3.0 at 40 C; the same in the presence of 0.1% Na-lauryl sulfate; the same in the presence of 0.166% cephalin; spores suspended in phosphate buffer at 40 C for 30 min.

With *n*-dodecylamine at 60 or 70 C, spore suspensions reproducibly are quantitatively converted to viable, heat-sensitive cells in 5 sec or less. The cells become slightly enlarged, are nonrefractile, have discharged their dipicolinic acid and mucopeptide, and are stainable. This practically instantaneous chemical germination is important evidence that in this system the prime germination event may not be obligately dependent on enzyme activity or biosynthesis of enzymes. Even energy-yielding reactions (Halvorson and Church, 1957*a*) appear not to be essential. It invites consideration of this initiating germination event as a physicochemical process. In this connection, a variety of enzyme poisons failed to prevent the optical density changes induced by *n*-dodecylamine. The inhibitors included: iodoacetic acid, 0.02 M; malonic acid, 0.01 M; sodium fluoride, 0.01 M; 2,4-dinitrophenol, 0.001 M; arsenous acid, 0.01 M; sodium azide, 0.01 M; ethyl alcohol, 10%;

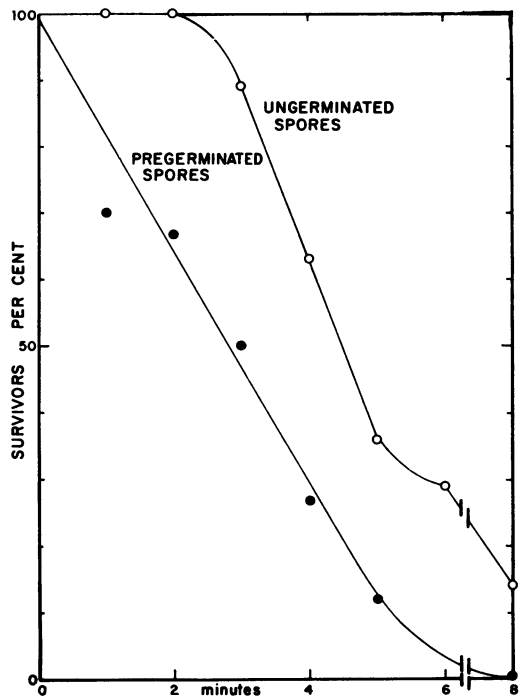

Fig. 5. Killing of ungerminated and pregerminated spores of *Bacillus megaterium* with 6 × 10^{-5} M *n*-dodecylamine; 0.066 M phosphate buffer, pH 8.0; temperature, 40 C. Pregerminated spores were prepared by exposing preheated spores to a mixture of L-alanine and inosine in 0.066 M phosphate buffer until complete germination was achieved, as described in the text. At the indicated times of exposure to the *n*-dodecylamine, a 3 × 10^{-4} dilution was made using 0.066 M phosphate buffer containing 0.166% cephalin.

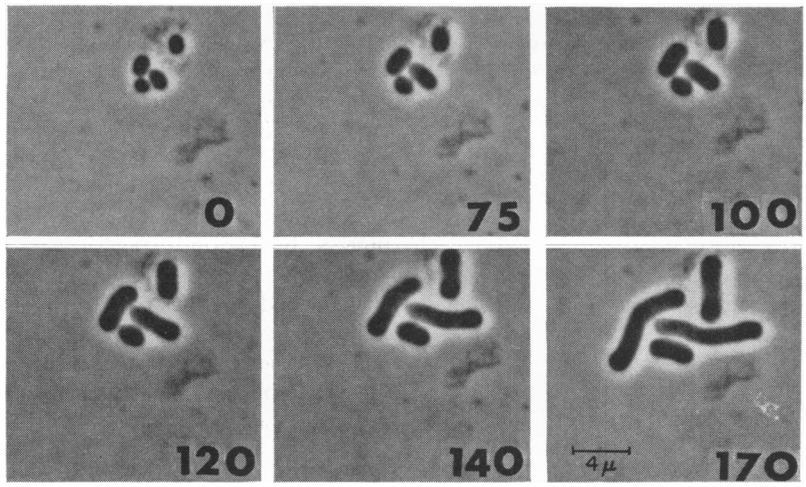

Fig. 6. Time lapse photomicrograph of the growth development of one field of spores of *Bacillus megaterium* germinated for 2.5 min with 6×10^{-5} M *n*-dodecylamine in 0.066 M phosphate buffer, pH 8.0; temperature, 40 C. The cells were promptly impinged on a membrane filter and washed with 0.066 M phosphate buffer containing 0.166% cephalin, then with buffer alone. All of the spores were nonrefractile at this point. The cells were suspended in nutrient broth, mounted on agar (Rode and Foster, 1960*a*), and a suitable field photographed at the minutes indicated.

phenol, 0.1%; hydrogen peroxide, 5 and 15%; sodium cyanide, 0.1 and 1.0%.

n-Dodecylamine and pregerminated spores. Other cationic surfactants induce changes in spores akin to germination, but the cells so formed fail to produce colonies. This situation was ascribed to the hypersensitivity of the germinated spores to those concentrations of the surfactants required to effect the initial germinative changes in the resting spores (Rode and Foster, 1960*c*). In view of the observation that alkyl amines were less lethal enough to allow recovery of viable germinated spores, an accurate assessment of the toxicity of *n*-dodecylamine for germinated spores had to be obtained as a prelude to other kinds of investigations. Fig. 5 shows that spores pregerminated physiologically with an L-alanine-inosine mixture were killed rapidly by *n*-dodecylamine. Although ungerminated spores treated with the same concentration of *n*-dodecylamine were killed at the same rate, this did not commence until after the first 2 to 3 min. The nonlethality of the alkyl amine during this early period in which this compound converts practically the entire population to heat-sensitive, nonrefractile cells, recoverable as colonies, is a demonstration of chemical germination.

Outgrowth of spores germinated with n-dodecylamine. A microscopic study of the metamorphosis of a spore to a vegetative cell is now possible for the first time without the use of physiological germinants. This was done by means of time lapse photomicrography employing the technique previously described. Fig. 6 shows that by this technique the swelling, elongation, and cell division which are induced by *n*-dodecylamine are indistinguishable from those occurring during physiological germination (Rode and Foster, 1960*c*).

Chemical changes. The following studies had the objective of determining whether changes analogous to those occurring in physiological germination take place during chemical germination with *n*-dodecylamine.

(1) Weight loss:—Three suspensions of spores of *B. megaterium* in 0.066 M phosphate buffer (pH 8.0) were compared: (i) a control in buffer alone, (ii) one containing 6×10^{-5} M *n*-dodecylamine, and (iii) one containing L-alanine (10^{-4} M) and inosine (1.4×10^{-4} M). The exposure time was 15 min for the *n*-dodecylamine, 30 min for the other two cases. The spores were weighed after centrifugation and drying at 100 C. The control spores in buffer suffered no detectable weight loss. The spores germinated with *n*-dodecylamine lost 45 to 55% of their dry weight (two experiments) and the spores ger-

TABLE 4

*Release of soluble constituents during germination of spores of Bacillus megaterium by n-dodecylamine**

Spore Constituent	Spores without *n*-Dodecylamine		Spores with *n*-Dodecylamine	
	Not solubilized	Solubilized	Not solubilized	Solubilized
	%	%	%	%
Spore solids:				
Expt no. 1	>99	<1	55	45
Expt no. 2			45	55
Calcium	3.0		0.4	2.6†
Protein:				
Expt no. 1		0.07		5.2
Expt no. 2				5.6
Dipicolinic acid:				
Expt no. 1	13.2	<0.3	<0.4	13.4
Expt no. 2				14.0
Amino acid:				
Total (acid hydrolysis)		0.7		11.4
Dialyzable fraction				7.0
Dialyzable fraction (acid hydrolysis)				7.5
Undialyzable fraction				0.4
Undialyzable fraction (acid hydrolysis)				3.7
Hexosamine (acid hydrolysis):				
Total		<0.1		2.1
Dialyzable fraction		<0.1		<0.1
Undialyzable fraction		<0.1		2.3

* Figures are based on the original weight of the spores. Spores suspended in phosphate buffer (pH 8.0); 6×10^{-5} M *n*-dodecylamine; temperature, 40 C. Experiment no. 1, 15 min; experiment no. 2, 30 min.

† By difference.

minated with alanine-inosine mixture lost 32%. The weight loss in the spores germinated with alanine-inosine is comparable to that recorded by Powell and Strange (1953).

(2) Solubilization of spore constituents:—The various analyses reported in Table 4 were performed on two 300-mg samples of spores, one suspended in *n*-dodecylamine solution, the other in buffer as a control. The changes in the control suspension were negligible. In the *n*-dodecylamine-treated spores, 85% of the spore calcium appeared to be released in soluble form. Also found in the supernatant was practically all of the spore dipicolinic acid, a considerable quantity of protein (5.5% of the spore weight), and an even larger quantity of amino acids (ninhydrin reaction). In the supernatant the bulk of the calcium was undoubtedly chelated by the dipicolinate; the ultraviolet absorption spectrum verified this assumption. All of the dipicolinic acid was dialyzable through cellophane, indicating that, at most, an insignificant fraction of the total could have existed in covalent combination with proteins or other high molecular weight substances (Rode and Foster, 1960*b*). Most of the amino acid fraction was dialyzable through cellophane, and from the fact that acid hydrolysis of the dialysate did not appreciably increase the ninhydrin-positive titer, evidently very little of this fraction existed as peptides. Approximately one third of the amino acids released were in sufficiently high molecular weight combination to be undialyzable. Acid hydrolysis resulted in a 9-fold increase in ninhydrin-reacting materials.

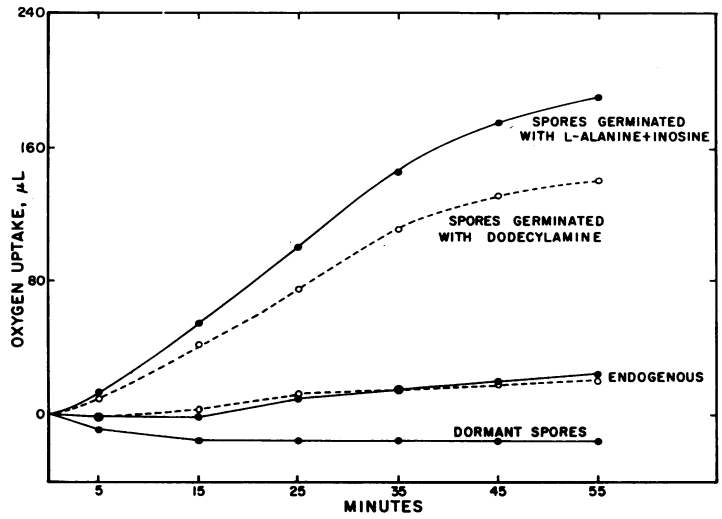

Fig. 7. Respiration of spores germinated with *n*-dodecylamine and with a mixture of L-alanine and inosine. Spores were germinated for 3 min in *n*-dodecylamine. The dodecylamine was then neutralized by the addition of 2 ml of 0.5% cephalin solution in 0.066 M phosphate buffer, pH 7.0. The spores were centrifuged and resuspended in 1.4 ml of 0.066 M phosphate buffer, pH 7.0. Of these suspensions, 1.3 ml were used in each Warburg respirometer: glucose, 0.72 mg per vessel; KOH in center well; temperature, 30 C. Physiologically germinated spores were obtained by exposing preheated spores of *Bacillus megaterium* to a mixture of L-alanine and inosine for 30 min at 37 C.

This fraction also contained virtually all of the hexosamine in the *n*-dodecylamine supernatant, and this was released by acid hydrolysis; hexosamine was not detectable in the unhydrolyzed supernatant. Characterization of the undialyzable fraction has not been completed, but a similarity and presumed identity with the mucopeptide isolated from bacterial spores by Strange and Powell (1954) is noted.

Thus, to the various features already found to be practically identical for physiological and chemical (*n*-dodecylamine) germination may be added the loss in spore weight and the compounds released in soluble form during germination.

Respiration. Germination by means of *n*-dodecylamine was accompanied by a prompt acquisition of respiratory activity (Fig. 7). Consistent with the viability data in Table 3, exposure of spores to *n*-dodecylamine for periods longer than 2 or 3 min progressively damaged the respiratory capacity of the spores. Ungerminated spores displayed negligible activity under these conditions. The rate of respiration of spores germinated with *n*-dodecylamine was maximal during the first 35 min. Experiments of this kind do not decide whether the enzymes involved preexisted in the spores (Halvorson and Church, 1957*b*), becoming activated through the *n*-dodecylamine treatment, or whether some enzyme synthesis occurred during the post-germination preparation and the equilibration time in the Warburg respirometer. A substantial equivalence of germination by *n*-dodecylamine and by a mixture of L-alanine and inosine, with regard to rate of glucose oxidation, is indicated in Fig. 7.

Sensitivity to physical and chemical stresses. Perhaps the most critical operational test of spore germination, namely, the conversion of a heat-resistant cell to a heat-sensitive cell, may not be unequivocal when applied to spores germinated with *n*-dodecylamine. It could be argued that the amine adsorbed on the spores enhances the lethal action of heat. The heat sensitivity of the treated spores would, therefore, only be apparent. However, in tests with a variety of other physical and chemical stresses which usually kill vegetative cells and germinated spores but which are innocuous to ungerminated spores, spores treated with *n*-dodecylamine behaved as though they were germinated and sensitive (Rode and Foster, 1960*d*). If the likelihood is remote that adsorbed *n*-dodecylamine augmented a lethal action of each of the diverse

treatments to a degree coincidental to their lethality for physiologically germinated spores, then it is logical to conclude that the *n*-dodecylamine-treated spores are, indeed, germinated, sensitive cells.

DISCUSSION

Considering the interplay among the factors required for optimal physiological germination of spores of this strain of *B. megaterium*, namely, a preheating followed by exposure to a mixture of L-alanine and inosine, the physiological process appears not to be simple. Moreover, the primary germinative incident is obscured by the multi-dependency. *n*-Dodecylamine circumvents all of those requirements; and in minute concentrations under conditions of pH and temperature which are physiological, this substance can effect germination of these spores at even faster rates than alanine and inosine. In a large number of the essential morphological, physiological, and chemical respects studied, a virtual identity of results has been demonstrated for the two procedures. Indeed, if other surface-active agents behave similarly (Rode and Foster, 1960*c*), one could make a case for an important role of these substances in germination under natural conditions, such as in the soil. A great many surface-active compounds are known to be synthesized by animal, plant, and microbial cells. A natural occurrence of such substances accounting for spore germination could be visualized even more readily than the fortuitous combination of alanine and inosine, considering their susceptibility to degradation or assimilation by the vastly larger numbers of vegetative microbial competitors invariably present in the microenvironment, as well as the needed conditioning of spores by a preheating. It may be that our distinction between physiological germination and chemical germination is arbitrary and premature in the particular case of surfactants, but operationally and conceptually it is useful inasmuch as a number of other types of chemicals are also known to evoke germination changes in spores (*unpublished data*).

At first sight germination by L-alanine-inosine mixtures and by *n*-dodecylamine appear to be quite different processes. However, the possibility should not be overlooked that when further details of the mechanism of initiation of the germination process are forthcoming, a basis for relating the two actions, perhaps even implicating them in a common step, may emerge. In any case, it seems certain that chemical germination with alkyl primary amines, even if shown to be dissimilar to physiological germination, will provide a valuable experimental approach to the nature of the structure and resistance of the ungerminated spore.

As mentioned in the experimental part, the relation between chain length and activity directs attention to the surface activity property of the alkyl primary amines as the one responsible for the initiation. Cationic surfactants are known to combine in salt formation with various anionic groups; conceivably, such groups are critical in the preservation of the dormant condition of the ungerminated spores. If the alkyl amines behave like the cationic quarternary ammonium surfactants, they would be rapidly and strongly adsorbed by some structure(s) in the spores preliminary to or as part of the germination events. This binding suggests an interaction between groups with opposite charges. Whether this adsorption process simultaneously displaces other groups with less affinity, or whether some other mechanism follows from the adsorption, can be decided only by more experiments. However, the site of the binding should now be accessible through the use of labeled surfactants (Rode and Foster, 1960*c*).

A second possible mechanism of action depends on the capacity of surfactants to wet surfaces and to solubilize lipids. If the ungerminated state of the spore is preserved by a barrier, which may be a coat, a membrane, or a zone situated outside of the core of the spore, any damage to the integrity of that barrier would allow exchange of dissolved substances between the internal parts of the spore and the external environment. The exchange would be subject to the selective permeability of the membrane surrounding the core, which is presumed to be equivalent to the plasma membrane. If lipid is indispensable for the integrity of the barrier, a localized mechanism of action of alkyl amines can be imagined. Apart from the evidence from thin section electron microscopy of the existence of structures and zones in the spore whose function is unknown, strong evidence for the existence of an unidentified barrier that is physical and tangible comes from the fact that spores may be germinated mechanically through abrasion with powdered

glass (Rode and Foster, 1960*a*). Also, although solutes penetrate the outer coats of the bacterial spore, the stainability of the core only after crushing led to the conclusion that a barrier shielding it had been broken (Black et al., 1960; Fitz-James, 1953).

One of the oldest plausible theories accounting for the resistance and the dormancy of spores is that of Lewith (1890) who suggested that the vital part of the spore is a vegetative cell in the anhydrous state, which is preserved by an impermeable, waterproofed barrier. The well known resistance to denaturation and the inertness of enzymatic proteins under conditions of anhydration, together with the data derived from refractive index measurements (Ross and Billing, 1957) are consistent with this hypothesis, and it has been invoked in recent years (Powell, 1957; Rode and Foster, 1960*a*).

If Lewith's hypothesis has substance, the protective barrier is impermeable to water. Until recently little evidence was available bearing directly on this point. Murrell and Scott (1958) measured the isotopic composition of spore water after equilibration with external water enriched with deuterium oxide. They concluded that less than 1% of the water in the spore failed to mix with external water and that their data did not support a theory based on exclusion of water. They noted that 1% of the spore water might not have been exchangeable. If this were in the core protected by a barrier, the substantial proportion of the spore volume comprised by the core would nevertheless allow for the possibility of a substantially anhydrous core. It is not clear how this technique could reveal the existence of regions in the spore (e.g., the core) which initially are anhydrous and not accessible to external water. Through an indirect technique these authors in another paper (Murrell and Scott, 1957) provide evidence that heat resistance of spores is largely dependent on the maintenance of some part of the spore contents in a relatively dry state.

Lewis, Snell, and Burr (1960) have introduced a new concept which at the same time embraces the anhydration theory and rejects a barrier to water permeability. They imagine a contractile, permeable membrane maintaining the anhydrous state by mechanical pressure upon the interior vegetative cell, through slow contraction of the cortex. However, this proposal is strictly conjectural.

Notwithstanding the inconclusiveness of the matter of accessibility of the core to water, most of the available evidence supports the idea of the spore core protected by a physical structure which functions as a barrier to the free penetration of solutes to the core and perhaps restricts the release of substances within the barrier.

That substances such as calcium dipicolinate and mucopeptide may comprise the barrier wholly or partly, must be considered. The events of chemical germination with *n*-dodecylamine suggest that the barrier is the locus of action. The almost instantaneous germination achievable with *n*-dodecylamine, taken together with the acquisition of respiratory activity without an apparent lag period, implies that the core is essentially a vegetative cell capable of an independent existence, and that the initiation of germination need not involve enzyme activity or biosynthesis. Neither of these ideas is new, but for the first time supporting evidence for them is available.

ACKNOWLEDGMENT

The capable assistance of Mr. Willard Lewis of the Electron Microscope Laboratory in the preparation of the electron micrographs is gratefully acknowledged.

SUMMARY

Several *n*-alkyl primary amines with carbon chain lengths of seven or more carbon atoms caused a marked reduction in the optical density of suspensions of spores of several species of the genus *Bacillus*. *n*-Dodecylamine was studied in detail in its action on spores of *Bacillus megaterium*. In the absence of a preheating, of alanine and inosine, and of any other organic substances, *n*-dodecylamine at neutral pH values and at temperatures from 37 to 70 C also causes several other changes. The spores lose refractility, become stainable with gentian violet, and discharge practically all of their dipicolinic acid and mucopeptide and most of their calcium. They also oxidize glucose and become sensitive to a variety of physical and chemical stresses. The heat-sensitive spores resulting from the action of *n*-dodecylamine on ungerminated heat-resistant spores were viable and produced colonies on nutrient agar. These spores are similar to germinated spores. The concepts of chemical germination and physiological germination are discussed. Possible mechanisms of action of the alkyl primary amines are presented in relation to the nature of the ungerminated spore.

REFERENCES

BLACK, S. H., R. E. MACDONALD, T. HASHIMOTO, AND P. GERHARDT 1960 Permeability of dormant bacterial spores. Nature, **185,** 782–783.

BROWN, W. L. 1957 Am. Inst. Biol. Sci., Publ. No. 5, 70–71.

ELSON, L. A., AND W. T. J. MORGAN 1933 A colorimetric method for the determination of glucosamine and chondrosamine. Biochem. J., **27,** 1824–1828.

EVANS, F. R., AND H. R. CURRAN 1943 The accelerating effect of sublethal heat on spore germination in mesophilic aerobic bacteria. J. Bacteriol., **46,** 513–523.

FALCONE, G., G. G. SALVATORE, AND I. COVELLI 1959 Mechanism of induction of spore germination in *Bacillus subtilis* by L-alanine and hydrogen peroxide. Biochim. et Biophys. Acta, **36,** 390–397.

FITZ-JAMES, P. C. 1953 The structure of spores as revealed by mechanical disruption. J. Bacteriol., **66,** 312–319.

HALVORSON, H. O. 1959 Symposium on initiation of bacterial growth. IV. Dormancy, germination, and outgrowth. Bacteriol. Rev., **23,** 267–272.

HALVORSON, H., AND B. D. CHURCH 1957*a* Intermediate metabolism of aerobic spores. II. The relationship between oxidative metabolism and germination. J. Appl. Bacteriol., **20,** 359–372.

HALVORSON, H., AND B. D. CHURCH 1957*b* Biochemistry of spores of aerobic bacilli with special reference to germination. Bacteriol. Rev., **21,** 112–131.

IMMERS, J., AND E. VASSEUR 1950 Influence of sugars and amines on the colorimetric hexosamine method of Elson and Morgan and its possible elimination. Nature, **165,** 898–899.

LAWRENCE, C. A. 1950 Mechanism of action and neutralizing agents for surface-active materials upon microorganisms. Ann. N. Y. Acad. Sci., **53,** 66–75.

LEWIS, J. C., N. S. SNELL, AND H. K. BURR 1960 Water permeability of bacterial spores and the concept of a contractile cortex. Science, **132,** 544–545.

LEWITH, S. 1890 Ueber die Ursache der Widerstandsfähigkeit der Sporen gegen hohe Temperaturen. Arch. exptl. Pathol. and Pharmacol., **26,** 341–354.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193,** 265–275.

MARTIN, H. H., AND J. W. FOSTER 1958 Biosynthesis of dipicolinic acid in spores of *Bacillus megaterium*. J. Bacteriol., **76,** 167–178.

MOORE, S., AND W. H. STEIN 1948 Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem., **176,** 367–388.

MURRELL, W. G., AND W. J. SCOTT 1957 Heat resistance of bacterial spores at various water activities. Nature, **179,** 481–482.

MURRELL, W. G., AND W. J. SCOTT 1958 The permeability of bacterial spores to water. Intern. Congr. Microbiol., 7th Congr. (Stockholm), 26.

PERRY, J. J., AND J. W. FOSTER 1955 Studies on the biosynthesis of dipicolinic acid in spores of *Bacillus cereus* var. *mycoides*. J. Bacteriol., **69,** 337–346.

POWELL, J. F. 1950 Factors affecting the germination of thick suspensions of *Bacillus subtilis* spores in L-alanine solution. J. Gen. Microbiol., **4,** 330–338.

POWELL, J. F. 1957 Biochemical changes occurring during spore germination in *Bacillus* species. J. Appl. Bacteriol., **20,** 349–358.

POWELL, J. F., AND R. E. STRANGE 1953 Biochemical changes occurring during the germination of bacterial spores. Biochem. J., **54,** 205–209.

RIEMANN, F. H., AND Z. J. ORDAL 1960 Studies on the germination of clostridial spores. Bacteriol. Proc., **1960,** 44.

RODE, L. J., AND J. W. FOSTER 1960*a* Mechanical germination of bacterial spores. Proc. Natl. Acad. Sci. U. S., **46,** 118–128.

RODE, L. J., AND J. W. FOSTER 1960*b* Induced release of dipicolinic acid from spores of *Bacillus megaterium*. J. Bacteriol., **79,** 650–656.

RODE, L. J., AND J. W. FOSTER 1960*c* The action of surfactants on bacterial spores. Arch. Mikrobiol., **36,** 67–94.

RODE, L. J., AND J. W. FOSTER 1960*d* Germination of bacterial spores with long chain alkyl amines. Nature, **188,** 1132.

ROE, J. H., AND B. S. KAHN 1929 The colorimetric determination of blood calcium. J. Biol. Chem., **81,** 1–8.

ROSS, K. F. A., AND E. BILLING 1957 The water and solid content of living bacterial spores and vegetative cells as indicated by refractive index measurements. J. Gen. Microbiol., **16,** 418–425.

STRANGE, R. E., AND J. F. POWELL 1954 Hexosamine-containing polypeptides in spores of *Bacillus subtilis*, *B. megaterium*, and *B. cereus*. Biochem. J., **58,** 80–85.