DIFFERENTIAL DIAGNOSIS OF *BACILLUS CEREUS*, *BACILLUS ANTHRACIS*, AND *BACILLUS CEREUS* VAR. *MYCOIDES*

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The purpose of this paper is to examine the adequacy of criteria which are commonly used to make a laboratory diagnosis of anthrax and to offer additional evidence on the close relationship existing between *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus cereus* var. *mycoides*. Certain erroneous concepts concerning the differentiation of these organisms have arisen and have gained acceptance to a degree which may preclude critical reexamination. Such fallacies have resulted largely from failure to recognize the transitional nature of the bacteria to which the laboratory tests were applied. There is no single characteristic which serves unequivocally to classify a gram-positive, sporeforming bacterium such as *B. anthracis*. From a practical standpoint, the ability of such an organism to produce anthrax when injected into or ingested by a susceptible animal is sufficient proof of its identity. A definition based solely on pathogenicity gives no clue to the natural origin of the species and reveals nothing of the other attributes of these pathogenic bacilli. The epidemiologist should be cognizant of these facts. The pathogen may arise from a natural population only under the directing influence of a specialized environment and bacilli capable of producing anthrax in the laboratory may not be of equal epidemiological significance in a natural environment.

Pathogenicity and the absence of motility have been the characteristics considered to be of greatest importance in the identification of *B. anthracis*. In recent years some laboratories have accepted mouse pathogenicity unsupported by properly selected corollary tests as diagnostic for the anthrax bacillus. This is unwarranted both in view of the convincing evidence which was presented by Grierson (1928) and Nordberg (1953), and the data given in table 5, that mice are susceptible to a high percentage of motile strains classified as *B. cereus*. Inadequate motility testing has delayed the recognition of other transitional forms. Undeserved emphasis has been placed on variable characteristics such as type of growth in gelatin, capsule formation, and hemolysis.

During the past fifty years various workers have studied the behavior of anthrax and anthrax-like bacilli and have described the characteristics of pathogenic and of nonpathogenic strains. The results have been confusing since some authors (Burrows *et al.*, 1949; Hagan, 1950; Leise *et al.*, 1954; Stein, 1943; and Ford, 1927) held that *B. anthracis* and *B. cereus* should be differentiated as species on the basis of biochemical, morphological, and pathogenicity tests, whereas Smith *et al.* (1952), Seidel (1954, 1956), and Brown *et al.* (1955) have contended that these organisms are merely variants of each other or of a common ancestor. Brown and co-workers have shown that motility can be induced in certain nonmotile cultures of the anthrax bacilli. These results strongly imply the presence in some anthrax bacilli of a latent genetic potential for motility. Such mutant cultures do not differ significantly from their parent cultures of *B. anthracis* except with respect to motility. Difficulties have been encountered in the repetition of the experiments on the induction of motility in *B. anthracis* by means of bacteriophage lysates. This has been discussed by Sterne and Proom (1957).

This paper attempts to define the present status of laboratory tests for the diagnosis of anthrax by reporting the writers' experience based on the examination of more than 250

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TABLE 1
Microscopic appearance of bacilli

Properties Studied	*Bacillus anthracis* (131 strains)	*Bacillus cereus* (115 strains)	*Bacillus mycoides* (70 strains*)
1. Size	0.9 μ to 1.3 μ by 2.9 μ to 5.2 μ	Same	Same
2. Chain formation			
Under 10 cells per chain	68	60	37
Over 10 cells per chain	69	55	33
3. End of cells	Square to subrounded	Same	Same
4. Spores	Oval, central to paracentral	Same	Same
5. Sporulation (in peptone broth)			
Under 24 hr	42	100	59
After 24 hr	92	15	11
6. Capsule formation (in inactivated mule serum). Read 6, 12, 18 hr			
Capsules formed	91	72	8*
No capsulation	46	43	62
7. Capsules observed (in cells isolated from animal tissue)	118	26	3
Not observed	19	72 Avirulent 17 Virulent (no capsule)	67

* Includes 8 nonrhizoid nonmotile dissociants.

cultures of *B. cereus*, *B. cereus* var. *mycoides*, and *B. anthracis* studied under similar conditions. Since they are especially significant for differential diagnosis, attention will be given to bacteriophage identification, the spontaneous loss of motility by motile species, and the production of both attenuation or increase in virulence by certain laboratory manipulations.

METHODS

The strains of bacilli used in this study were obtained from various laboratories throughout the world, not more than 20 strains originating from any one region. Strains including 115 labeled *B. cereus*, 38 of *B. cereus* var. *mycoides*, and 137 of *B. anthracis* were employed. In addition, 32 nonrhizoid dissociants of *B. cereus* var. *mycoides* were used in the motility and pathogenicity tests. Of these, 27 were isolated from our laboratory strains by the methods described by Gordon (1940) and 5 were obtained from the Nathan R. Smith collection. The cultures of *B. anthracis* included both freshly isolated strains from human and animal sources and those from old laboratory stock cultures.

The animals used for the experiments in patho-

TABLE 2
*Motility studies**

Organism	Media				
	Hanging drop	Edwards and Bruner	Gard's swarm	Fischer's transmigration	Difco motility
Bacillus anthracis					
Motile	1†	1†	0	0	0
Nonmotile	122	122	122	122	122
Bacillus cereus					
Motile	89	115	77†	97	60
Nonmotile	8	0	0	0	0
Bacillus mycoides					
Motile	17	29‡	30‡	29‡	5
Nonmotile	21	9	8	9	33
Bacillus mycoides (nonrhizoid)					
Motile	8	7‡	7‡	7‡	Not tested
Nonmotile	26	25	25	25	

* Figures represent number of strains; negative tests held at 37 C for 14 days.

† One strain sent to us as *B. anthracis* but as yet unclassified apparently gives off occasional motile variants.

‡ Motility only slight at 48 hr.

TABLE 3

Cultural and bacteriophage studies on bacilli

	Bacillus anthracis (122 strains)	*Bacillus cereus* (115 strains)	*Bacillus mycoides* (38 strains)	*Bacillus mycoides* (32 dissociant strains)
Appearance on nutrient agar (37 C)				
Anthrax-like	106	26	rhizoid	16
Cereus-like	16	89		16
Growth in heart infusion broth (18 hr)				
Floccular sediment, no turbidity, no pellicle	78	16	4	26
Granulo-floccular sediment, granular turbidity, no pellicle	44	89	34	6
Appearance in gelatin (20 C)				
"Inverted pine tree"	21	41	13	13
No pinetree	101	74	25	19
Liquefaction of gelatin (20 C)				
Liquefied within 4 days*	32	67	18	13
Not liquefied within 4 days	90	48	17	19
Reduction of methylene blue (37 C)				
In 24 hr	7	69	36	19
In 72 hr	74	102	38	31
In 96 hr	122	115	38	32
Reduction of litmus milk (37 C)				
Within 48 hr	0	36	7	15
48 hr to 3 weeks	122	61	31	17
Lysis by bacteriophage				
γ	122	0	0	8
W	122	3	0	6
201‡	122	10	8	8
Hemolysis (on 5% rabbit blood for 48 hr)				
Nonhemolytic	75‡	26§	0	26
Hemolytic	45¶	89	38	6
Lecithinase (Action on egg-yolk media)	(89 Strains tested)	(97 Strains tested)	(15 Strains tested)	(9 Strains tested)
Positive	19	89	15	3
Negative	70‡	8	0	6
Growth on De Angelis medium (Ferric chloride-Gelatin test)	(10 Strains tested)	(10 Strains tested)	(4 Strains tested)	(Not tested)
Positive	6	3	2	—
Negative	4	7	2	—
Growth on penicillin agar (6 i.u./ml)	(13 Strains tested)	(12 Strains tested)	(10 Strains tested)	(6 Strains tested)
Inhibited	12	3	2	5
Not inhibited	1	9	8	1

* An arbitrary line was drawn after 4 days of incubation; by 21 days all cultures attacked gelatin.

† Only 12 strains of *B. anthracis*; 10 of *B. cereus*; 8 of *B. mycoides* and the nonrhizoid dissociants were tested.

‡ All virulent strains for mice.

§ Including 13 strains virulent for mice.

¶ Including 13 avirulent strains for mice.

TABLE 4

Biochemical and nutritional characteristics of bacilli

	Bacillus anthracis (122 strains)		*Bacillus cereus* (115 strains)		*Bacillus mycoides* (70 strains)		*Bacillus mycoides* (38 variant strains)	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Glucose	122	0	115	0	70	0	38	0
Sucrose	122	0	115	0	70	0	38	0
Lactose	1	121	0	115	0	70	0	38
Dulcitol	0	122	0	115	0	70	0	38
Salicin								
Within 24 hr	28	94	78	37	52	18	19	29
After 48 hr	35	87	109	6	62	8	33	5
Xylose	0	122	0	115	0	70	0	38
Maltose	110	12	95	20	47	23	29	9
Mannitol	0	122	0	115	0	70	0	38
Arabinose	0	122	0	115	0	70	0	38
Trehalose	120	2	114	1	70	0	38	0
Sorbitol	0	122	0	115	0	70	0	38
Inositol	0	122	0	115	0	70	0	38
Adonitol	15	107	25	90	41	29	23	15
Glycerol	77	45	101	14	62	8	36	2
Raffinose	0	122	0	115	0	70	0	38
Rhamnose	0	122	0	115	0	70	0	38
Galactose	0	122	0	115	0	70	0	38
Mannose	0	122	0	115	0	70	0	38
Catalase	122	0	115	0	70	0	38	0
Hydrolysis of starch	122	0	115	0	70	0	38	0
Hydrolysis of casein	122	0	115	0	70	0	38	0
H_2S production	0	122	0	115	0	70	0	38
Nitrate reduction	95	27	81	34	59	11	20	18
Indole formation	0	122	0	115	0	70	0	38
Simmon's citrate	119	3	108	7	68	2	37	1
Acetylmethylcarbinol	120	2	111	4	69	1	37	1
Growth on NH_4 agar	121	1	115	0	70	0	38	0
Growth on 1% tyrosine agar	122	0	115	0	70	0	38	0
Growth on 1% glucose agar	122	0	115	0	70	0	38	0

TABLE 5

Pathogenicity studies

		Bacillus anthracis	*Bacillus cereus*	*Bacillus mycoides*	*Bacillus mycoides* (nonrhizoid)	*Bacillus megaterium*	*Bacillus subtilis*	Broth†
Mice	No. strains tested*	120	63	27	12	5	5	5
	No. strains pathogenic	107	26	3	0	0	0	0
	Pathology typical of anthrax	104	25	3	3	0	0	0
Guinea pigs	No. strains tested	47	26	27	12	5	5	5
	No. strains pathogenic	41	7	3	0	0	0	0
Rabbits	No. strains tested	42	24	27	12	5	5	5
	No. strains pathogenic	34	0	0	0	0	0	0

* 0.2 ml of an 18 hr heart infusion broth (HIB) culture given subcutaneously.

† Represent 2 aliquots from different (5) heart infusion broth tubes.

genicity consisted of rabbits of mixed breed and sex, weighing from 6 to 8 lb; guinea pigs of mixed breed and sex, weighing 200 to 300 g; and full-grown white Swiss mice. All animals were maintained on antibiotic-free feed.

Detailed descriptions of the procedures followed in the morphological, cultural, and biochemical studies of the bacilli are given in the experimental section, but they were, in general, those described by Smith *et al.* (1952) and by Stein (1944). Pathogenicity was determined either by the methods of Nordberg (1953) or by simulating the dosage and route of administration most frequently used in various laboratories concerned with the diagnosis of anthrax, as judged by a survey of the recent literature. Cultures were tested for susceptibility to phage by the methods of Brown and Cherry (1955).

EXPERIMENTAL RESULTS

Microscopic examination. All strains of bacilli were examined after staining by Gram's method and by methylene blue. Capsule formation was determined in wet and dry India ink preparations, and by the Hiss copper sulfate method. For this purpose organisms were grown in inactivated mule-serum broth at 37 C; cultures were kept on a shaker and examined at 6, 12, and 18 hr. In addition, smears were taken from animal tissues (spleen, liver, and heart blood) and examined for the presence of encapsulated organisms. The presence of fat vacuoles was tested by the procedures of Burdon *et al.* (1942). The presence of spores was ascertained by the method of Smith *et al.* (1952) and by Dorner's technique.

The results of this study are tabulated in table 1, where it can be seen that no significant microscopic differentiation could be made between *B. anthracis*, *B. cereus*, and *B. cereus* var. *mycoides*.

Motility. Motility was investigated in detail by the following techniques: hanging drop, observation for as long as 21 days in motility medium (Difco), in Edwards' and Bruner's (1942) semisolid medium, in Nordberg's (1951) modification of Gard's medium, and in Fischer's (Nordberg, 1951) egg transmigration medium. The results of these tests (table 2) showed that none of the 122 strains of *B. anthracis* was motile, although one additional strain as yet unclassified by us, apparently gives off an occasional motile variant. All of the 115 strains of *B. cereus* tested appeared motile in the semisolid medium of Edwards and Bruner and in Gard's swarming medium, while only 89 appeared actively motile in the hanging drop, 8 strains being either sluggishly motile or nonmotile. On motility agar (Difco), 41 of the motile strains, including 19 strains of *B. cereus* subsequently found pathogenic for white mice, appeared nonmotile. Of 38 strains of *B. cereus* var. *mycoides* which were examined microscopically for motility, 21 appeared nonmotile and 17 motile after incubation, both at 25 and 37 C for 48 hr. None of these strains appeared more than slightly motile in the other test media. Of the nonrhizoid dissociants, 2 of which were received from Dr. Ruth E. Gordon, the remainder being isolated in this laboratory by the technique of Gordon (1940), 7 appeared motile; 25 strains, including 7 received from Dr. Gordon and the remainder isolated elsewhere, appeared nonmotile in all examinations.

Cultural characteristics. (1) Appearance on solid medium:—Organisms were studied for their colonial and morphological appearance on heart infusion agar and upon nutrient agar plates. Streak plates were made and colonies were observed at 6, 18, and 48 hr. Typical colonies of *B. anthracis* and *B. cereus* had a cuneiform appearance when observed under oblique lighting. However, typical colonies of *B. anthracis* were viscid in consistency, whereas the colonies of *B. cereus* were not adherent when stabbed with a needle. As shown in table 3, the colonies of 106 of 122 strains of *B. anthracis* showed typical appearance. Including 5 strains which resembled vaccine strains described by Sterne (1937), 16 could not be distinguished from *B. cereus* by colonial appearance. Of the 115 strains of *B. cereus* tested (table 3), 89 consisted of typical rough colonies (Smith *et al.*, 1952); colonies of 26 strains, 6 of which were pathogenic for guinea pigs, were indistinguishable from *B. anthracis*. All 38 typical strains of *B. cereus* var. *mycoides* produced spreading, filamentous, tangled strands of growth which were difficult to remove from the agar surface, whereas colonies of 32 nonrhizoid dissociants were usually similar to those of *B. cereus* and *B. anthracis*, in their cuneiform appearance.

(2) Growth in broth:—Heart infusion broth and nutrient broth were inoculated from 18-hr cultures. The characteristics of growth in stagnant cultures are tabulated in table 3. It can be seen that *B. cereus* and *B. cereus* var. *mycoides* usually produced a granular turbidity while *B. anthracis* more frequently produced a floccular sediment without turbidity. However, it is apparent that

B. anthracis cannot be differentiated with certainty by this characteristic.

(3) Growth in gelatin:—Inoculated tubes of nutrient gelatin were observed daily for a period of 3 weeks. For tabulation of results (table 3) an arbitrary line was drawn after 4 days of incubation. Clear-cut differentiation of these species could not be drawn, although it can be stated that *B. anthracis* has a tendency to liquefy gelatin more slowly than does *B. cereus*. As indicated by the experiments, no reliability could be placed on the differentiation of *B. anthracis* from related species by means of the classical "inverted pine tree" appearance.

(4) Growth of McGaughey and Chu's medium:—The production of lecithinase by the organisms was determined by the method of McGaughey and Chu (1948), and the results are listed in table 3. The results indicate that most strains of *B. cereus* produce lecithinase while most strains of *B. anthracis* failed to give a positive test. It is obvious that this test is unreliable to the extent that overlapping of results occurred.

(5) Growth on penicillin agar:—Plates of heart infusion agar containing 6 units of penicillin G (Wyeth) per ml were prepared, inoculated with a loopful of growth from agar slant cultures (18 hr) of the organisms tested, and the plates were incubated at 37 C for 18 hr before examination. A total of 13 strains of *B. anthracis*, 12 of *B. cereus*, and 10 of typical *B. mycoides*, in addition to 4 nonrhizoid dissociants were studied. The results of this study are recorded in table 3. One disadvantage of this technique (Jensen, J., 1957, *personal communication*), is the fact that secondary growth on the part of *B. anthracis* has led to erroneous results in the diagnosis of the anthrax organism.

(6) Ferric chloride-gelatin test of De Angelis.—The medium and reagents were made up according to the specifications of De Angelis (1937) and his procedures were followed. Included in the test were 10 strains of *B. anthracis* fully virulent for rabbits, 10 strains of *B. cereus*, and 4 strains of *B. mycoides*. The results are shown in table 3. According to De Angelis, *B. cereus* strains gave consistently negative results. However, in our studies, 3 motile strains gave positive reactions consistent with those expected for *B. anthracis*. On the other hand, 4 strains of the anthrax bacilli gave negative reactions both in the direct and indirect tests. Since these results offered no promise that the test was of differential value, it was not used further.

(7) Hemolysis.—The methods of Stein (1944) were followed to determine the hemolytic activity of cultures. Since the results obtained in blood broth paralleled those obtained on blood agar plates, only the latter were tabulated (table 3). No sharp distinction could be made between the groups studied.

Biochemical reactions. (1) Reducing ability:—The action of the cultures on litmus milk was determined, and reduction of methylene blue in the medium described by Smith *et al.* (1952) was recorded. Table 3 shows the results obtained when the tubes of methylene blue and litmus milk incubated at 37 C were examined for reduction.

(2) Fermentation reactions:—Either infusion or tryptone broth was used as a basal medium for fermentation tests. Except as indicated below, substrates for all tests (table 4) were inoculated, incubated, and read according to the procedures described by Smith *et al.* (1952).

Catalase production was estimated by the addition of approximately 1 ml of hydrogen peroxide (3 per cent) to the surface of a slant of tryptone yeast-extract-glucose medium which had been inoculated and incubated at 37 C for 24 hr.

Tests were made for hydrogen sulfide by suspending strips of lead acetate paper in the mouths of tubes containing infusion broth or nutrient agar slants which had been inoculated with the test organisms and incubated at 37 C. The ability of the organisms to grow on synthetic agar of Smith and co-workers was determined.

An analysis of these results agrees with the finding of Smith *et al.* (1952) who found no marked biochemical differences among the species.

Pathogenicity studies. The virulence of representative strains was tested by growing cultures in heart infusion broth at 37 C for 6 to 18 hr and inoculating 0.2 ml subcutaneously or intraperitoneally into mice, guinea pigs, and rabbits. In order that a more valid comparison of pathogenicity could be made, the size of the dose was selected to conform to that most commonly used by other workers. Whereas a larger dosage was used, it has been the authors' experience that 0.02 ml of a 6-hr broth culture of virulent strains produced infection. The changes considered typical of anthrax were the presence of a gelatinous exudate at the site of injection and enlarged

TABLE 6

Pathogenicity studies: Culture passed in blood-dextrose medium and 0.2 ml given subcutaneously

	No. Strains*	*Bacillus anthracis*	*Bacillus cereus*	*Bacillus mycoides*	*Bacillus mycoides* (Dissociant)	*Bacillus megaterium*	*Bacillus subtilis*	Broth
Mice	Tested	18	28	12	12	5	5	5
	Pathogenic before passage	8	0	0	0	0	0	0
	Pathogenic after passage	18	28	9	12	0	0	0
	Typical gross pathology	18	27	9	12	0	0	0
Guinea pigs	Tested	18	28	12	12	5	5	5
	Pathogenic before passage	0	0	0	0	0	0	0
	Pathogenic after passage	18	21	9	12	0	0	0
Rabbits	Tested	18	28	12	12	5	5	5
	Pathogenic before passage	0	0	0	0	0	0	0
	Pathogenic after passage	18	14	5	9	0	0	0

* Duplicate animals used in each test.

† Edema; dark-red enlarged liver and spleen containing encapsulated organisms.

dark red liver and spleen. These pathological changes were most pronounced in animals dying 30 hr or longer after inoculation. Table 5 shows the results of the experiments.

In order to enhance the virulence of nonpathogenic strains or strains virulent for mice only, heart infusion broth containing 10 per cent of 5-day old rabbit blood and 10 per cent glucose was prepared and the pH adjusted to 7.4. The 28 strains of *B. cereus* including 10 freshly isolated from the air, 5 strains of *B. subtilis*, 5 of *B. megaterium*, 12 nonrhizoid variants of *B. cereus* var. *mycoides*, 12 of *B. cereus* var. *mycoides*, and 18 of *B. anthracis* (10 of which were avirulent and 8 virulent for mice only) were grown for 18 hr in blood-glucose broth. The cultures were streaked on plates of blood-glucose agar and incubated at 37 C for 3 hr. Colonies, which were just visible at that time, were scraped off with the wire loop and reinoculated on a second plate and so on through the third passage at which time the 3-hr growth was suspended in 8 to 10 ml of heart infusion broth. Mice, guinea pigs, and rabbits were inoculated with up to 0.2 ml of this suspension. Controls consisted of uninoculated glucose blood broth and of the washings from blood glucose plates which had been flooded with sterile heart infusion broth and subjected to the same manipulations as were the test materials. The results of attempts to enhance virulence by growth on blood media are presented in table 6. At autopsy all animals susceptible to infection showed the gross pathology of anthrax and encapsulated organisms were demonstrated in one or more organs.

Bacteriophage identification. For this work the following 3 bacteriophages were used: γ, W, and 201. Undiluted phages of the following titers were applied directly to the test cultures inoculated onto the surface of plates of heart infusion agar: γ, 2×10^8; W, 1×10^7; and 201, 3×10^5 plaque-forming particles per ml of lysate. The results of these experiments as shown in table 3 indicate that γ phage gives clear differential lysis of the anthrax bacilli and lysed only 4 nonrhizoid strains of *B. cereus* var. *mycoides*, whereas W and 201 phages cannot be used for species differentiation.

DISCUSSION

From the results we concur in the views of Smith *et al.* (1952) and Brown and Treece (1956) that *B. anthracis* is a pathogenic variant of *B. cereus*. Absence of motility, susceptibility to certain phages and pathogenicity of small inocula for rabbits constitute the main differential characteristics of *B. anthracis*. The fact that a recognized (Breed *et al.*, 1957) variant of *B.*

cereus, such as *B. mycoides*, can dissociate into nonrhizoid, nonmotile variants which in turn, by passage on suitable media, become virulent and otherwise indistinguishable from anthrax, further substantiates this view.

The W phage described by McCloy (1951) and the mutant phage, γ, which was derived from it (Brown and Cherry, 1955) were very helpful in making an identification of anthrax bacilli (table 3) when the results were combined with those of motility and pathogenicity tests. It should be noted that both the γ and the W phages lysed some strains of nonrhizoid *B. mycoides*. Since such cultures could be distinguished from anthrax bacilli only by a lesser degree of pathogenicity, their susceptibility to phage is not undesirable. It was interesting to note that none of the 38 rhizoid strains of *B. mycoides* was lysed by either W or γ phage. From table 3 it is apparent that both phages, γ and W, lysed all (129) cultures of *B. anthracis* on which they were tested, whereas γ failed to act on any of the 118 strains of *B. cereus*, and W lysed only 5 strains. In comparison, phage 201 of Smith *et al.* (1952) had little specificity for the anthrax bacilli. Thus, γ and W phages have proved to be valuable tools for the study of unknown cultures. One of the writers (M.D.M.) found the γ phage to be of substantial assistance in the study of organisms obtained from cattle during a recent anthrax outbreak.

Dr. G. Seidel, Humboldt University, Berlin, has kindly allowed the author to read his manuscript, "Zur bakteriologischen Differential Diagnose des Milzbrandes," which he will publish in the near future. On the basis of a study of 47 strains of *B. anthracis* and 97 cultures of closely related organisms, Seidel called attention to the following characteristics as being most helpful for the identification of *B. anthracis*: determination of motility in transgression medium (Fischer), or on the swarming medium of Gard, observation of colonial growth characteristics, and of growth on Petragnani's medium containing malachite green and thionin. On the latter medium, all cultures of *B. anthracis* grew typically, whereas only 16.5 per cent of the anthrax-like strains appeared typical of *B. anthracis*. Some nonanthrax cultures produced colonies which could not be differentiated from those of typical *B. anthracis*. The Ascoli thermoprecipitation test was considered to be very helpful in the diagnosis of anthrax in decomposed or highly contaminated animal bodies. However, Seidel pointed out that a positive result depends to a large extent upon the quality of the serum which is employed and that some nonanthrax bacilli (15 of 97) gave positive results. Mouse pathogenicity was not a dependable diagnostic characteristic since 19 of 47 strains labeled as *B. anthracis* failed to kill mice while 10 of the 97 strains which were anthrax-like produced lethal infections.

Jensen and Kleemeyer (1953) compared 50 strains of *B. anthracis* and 43 strains of anthrax-like bacteria with respect to the usual differential tests for diagnosis and for type of growth on penicillin agar (Perlschnurtest). The 50 strains were completely typical of *B. anthracis* in type of growth on penicillin agar although 18 of these behaved atypically with respect to one or more of the usual diagnostic criteria. None of 43 anthrax-like cultures grown on penicillin agar resembled *B. anthracis*, although 23 strains had 2, 13 had 3, and 1 strain had 4 diagnostic characteristics in common with the causative agent of anthrax. Since the test is rapid and specific it was concluded that growth on penicillin agar, together with the type of growth in gelatin stabs (inverted pinetree), are among the most useful diagnostic aids for the identification of the anthrax bacilli. Their enthusiasm for the growth in gelatin stab tubes is not shared by the writers nor by Seidel (1954).

We disagree with Scatterday *et al.* (1954), Croft and Stegmiller (1952), Gordon *et al.* (1954), Leise *et al.* (1954), and other investigators that the mouse can be used alone as the test animal in making an identification of *B. anthracis* even when supported by the results of other commonly used diagnostic tests. Mice may be used for screening unknown cultures for pathogenicity, but all positive cultures should be retested by inoculating rabbits or guinea pigs. This opinion is based on the fact that 42 per cent of the strains of *B. cerus* studied by the authors were pathogenic for mice in doses of 0.2 ml and that 12.3 per cent of these were also virulent for guinea pigs. These figures agree very closely with those of Nordberg (1953). Thus, it appears that the use of mice alone for animal differentiation of *B. anthracis* may result in false identification almost as often as in correct identification. We recognize that from the standpoint of routine laboratory diagnosis experienced workers make a correct identification of *B. anthracis* based on mouse pathogenicity aided by a small number of well

selected confirmatory tests. However, it is disturbing that many workers who have reported the isolation of *B. anthracis* have failed to describe the diagnostic criteria which were used. It appears that the somewhat arbitrary and noncritical acceptance of mouse pathogenicity, nonmotility, a certain type of growth in either gelatin or on solid media as characteristic of *B. anthracis*, has impeded the recognition of genetic relationships existing between *B. cereus*, *B. anthracis*, and *B. cereus* var. *mycoides*. The induction of motility in typically nonmotile strains of *B. anthracis* and the relative ease with which some mouse pathogenic cultures of *B. cereus* and *B. cereus* var. *mycoides* could be rendered virulent for guinea pigs and rabbits (table 6) emphasized the blending of characteristics of these organisms. Vincent (1898) reported that bacilli, presumably *B. cereus* and similar species, when placed within the peritoneum of guinea pigs by means of collodion sacs and successively transferred from one animal to another, resulted in bacilli causing all typical symptoms of anthrax. These organisms became encapsulated, were nonmotile and fully virulent in small doses for mice, guinea pigs, and rabbits; whereas their progenitors are all nonencapsulated, motile, and avirulent.

Some consideration must be given to the methods of testing for motility. The authors have found that a large number of *B. cereus* strains which were motile in Gard's, Edwards' and Bruner's, and Fischer's media appeared nonmotile in motility agar (Difco). This is due to the greater viscosity of this medium and to the turbidity which makes it difficult to determine the presence of light-spreading subsurface growth (table 2). Of 115 cultures, 11 were nonmotile when examined microscopically. These observations supported the findings of Seidel (1954), Brown and Treece (1956) and of Nordberg (1951, 1953) who emphasized the superiority of "transmigration" and of other semisolid media for the determination of motility. The semisolid medium described by Edwards and Bruner (1942) has proved very satisfactory in the hands of the writers when used either as a stab culture or as a swarm plate. Many authors, McFarland (1898), Hueppe and Wood (1889), Smith *et al.* (1952), Clark (1937) and more recently, Seidel (1954) have reported on nonmotile *Bacillus*. Those described by Grierson (1928) as *B. anthracoides*, by Gordon (1940) and Smith *et al.* (1952) as nonrhizoid dissociants of *B. mycoides*, by Seidel (1954) as an old laboratory strain of *B. cereus*, resembled the anthrax-like strains reported on in this paper.

The "typical" anthrax bacillus might be described as a gram-positive rod (in which spores and fat vacuoles are not numerous after 2 days incubation at 30 C), that produces viscid, cuneiform colonies made up of long tangled chains of nonmotile organisms; growth in broth occurs as a stringy sediment beneath a clear supernatant; virulent for rabbits when injected subcutaneously in doses of 0.2 ml of an 18-hr broth culture; and the cells are susceptible to lysis by γ phage.

"Typical" colonies of *B. cereus* appeared moist, nonviscid, and of such consistency that a loop could be passed easily through them, the cells appearing identical to *B. anthracis* except for motility and nonpathogenicity for rabbits and usually for guinea pigs; they were not susceptible to γ and W phages.

Between these two groups lie a whole range of intermediate bacilli encompassing the so-called "avirulent" nonmotile strains of *B. anthracis*, cultures of *B. anthracis* which are weakily virulent (mice only) such as some vaccine strains, and *B. cereus* strains which are sluggishly motile but virulent for mice and guinea pigs. Such characteristics as encapsulation of organisms freshly isolated from animal tissues, slow reduction of methylene blue, slow fermentation of salicin, absence of lecithinase, failure to hemolyze red blood cells, and characteristic growth on penicillin agar served as useful confirmatory tests for "typical" anthrax bacteria, but an appreciable number of *B. cereus* cultures have similar characteristics.

As discussed by Stein (1944) pathogenicity appeared to be connected in some inexplicable way with certain "anthrax-like" attributes such as the absence of hemolysins and of lecithinase, delayed salicin fermentation and reduction of methylene blue, wherein the more pathogenic the organism the more "anthrax-like" the tests. It sometimes happened that when a strain of *B. anthracis*, virulent for mice only, was transferred rapidly on a blood-glucose medium it was found to have altered characteristics, e. g., diminished zone of hemolysis and a negative test for lecithinase. More work should be done to prove or disprove these observations of artificially selected loss variations correlated with increased pathogenicity.

On the basis of our experiments as well as

those of others, it is felt that the nomenclature suggested by Smith, Gordon, and Clark, namely, *B. cereus* var. *mycoides* and *B. cereus* var. *anthracis* is appropriate. In contrast to Breed *et al.* (1957) in which *B. anthracis* appears as a separate species and *B. mycoides* as a variety of *B. cereus*, the authors indicate that the former would be described more accurately as a pathogenic variety of *B. cereus*. The authors point out that "pathogenicity is a variable character in these two species and that academically they should be classified as variants of *Bacillus cereus*, the stable parent form." There is certainly as much justification for designating the anthrax bacillus a pathogenic variety of *B. cereus* as for giving varietal status to *B. mycoides* by reason of its colonial variations. The degree of pathogenicity and motility in the so-called *B. anthracis* organisms certainly varies in stock cultures and is not a reliable, stable characteristic upon which to base a species separation. Indeed, there is now considerable evidence indicating that *B. anthracis* may exist, as a result of either natural or artificial processes or by a combination of these, in any of the following forms: pathogenic, nonmotile; pathogenic, motile; nonpathogenic, nonmotile; and nonpathogenic, motile. The first of these is the typical anthrax bacillus and the last is *B. cereus*.

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SUMMARY

A critical evaluation was made of the techniques commonly employed for the laboratory diagnosis of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus cereus* var. *mycoides*. The results were compared when large numbers of strains representing each species was employed. Evidence has been presented which strongly indicates that absence of motility, susceptibility to certain phages (especially γ), and pathogenicity of small inocula for rabbits constitute the main differential characteristics of *B. anthracis* when compared with those of other closely related gram-positive bacilli.

Attention was directed to the significance, for the study of genetic development, of the artificial selection of pathogenicity in *B. anthracis* resulting from serial passage on blood-glucose media. It was concluded that the names *Bacillus cereus*, *Bacillus cereus* var. *anthracis*, and *Bacillus cereus* var. *mycoides* furnish the most appropriate taxonomic description of these organisms.

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