CHEMICAL COMPOSITION AND HEAT RESISTANCE OF SOME AEROBIC BACTERIAL SPORES[1]

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ABSTRACT

WALKER, HOMER W. (Iowa State University of Science and Technology, Ames), JACK R. MATCHES, AND JOHN C. AYRES. Chemical composition and heat resistance of some aerobic bacterial spores. J. Bacteriol. **82**:960–966. 1961.—Analyses of spores of *Bacillus* species for nitrogen, carbohydrate, dipicolinic acid, and phosphorus showed little correlation with heat resistance. However, as the molar concentration of magnesium increased in relation to dipicolinic acid and calcium concentrations, heat resistance generally decreased. Analyses of several batches of spores indicated that this relationship between calcium, magnesium, and dipicolinic acid did not always hold true. Therefore, while these materials apparently play an important role, other factors need to be included before a full explanation of thermal stability of spores can be made.

The bacterial spore is noted for its ability to survive temperatures that ordinarily kill vegetative cells. This resistance has been of considerable importance in the preservation of foods, sterilization of bacteriological media, and in other areas in which the elimination of microbial life is necessary. Several explanations for the ability of the spore to exist under these adverse conditions have been made on a chemical basis.

Dipicolinic acid (DPA) has been found to constitute from 5 to 15% of the dry weight of the spore (Powell, 1953; Perry and Foster, 1955). Apparently, accumulation of certain levels of this compound is necessary before heat resistance exceeding that of the vegetative state occurs (Church and Halvorson, 1959; Hashimoto, Black, and Gerhardt, 1960). Upon germination of the spore, DPA is released with a loss of heat resistance (Powell, 1953; Woese and Morowitz, 1958).

Cations such as calcium, magnesium, and manganese have also been implicated with DPA in producing and maintaining thermostability. Curran, Brunstetter, and Myers (1943) have shown by spectrochemical analyses of the ash of six organisms that spores contain substantialy more calcium, copper, and manganese, but less potassium and phosphorus than do vegetative cells. Lechowich and Ordal (1960) reported that, as the DPA content and cation content increased in spores of *Bacillus coagulans*, the thermal resistance of the spore increased.

Some relationship between phosphorus content and resistance has been reported also. For example, the phosphorus content of the protein of mesophilic spore formers has usually been found to be higher than that found in thermophiles (Sobotka and Luisada-Opper, 1957). In addition, an increase in phosphate concentration in the sporulation medium for *B. coagulans* reduced thermal resistance (Amaha and Ordal, 1957). El-Bisi and Ordal (1956) suggested that phosphate interferes with the complexing of protein and calcium, thus decreasing the resistance of spore protein to denaturation. Curran (1957) has reviewed the information dealing with mineral requirements for sporulation and has indicated that the specific function of minerals in sporulation and thermostability has not yet been well explained. The present study was undertaken to analyze for certain components of spores of *Bacillus* species and to determine if any relationship between these constituents and heat resistance is evident.

MATERIALS AND METHODS

Cultures of the following organisms were obtained from the Department of Bacteriology,

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Iowa State University: *B. polymyxa* 1A39, *B. megaterium* 1A28, *B. megaterium* 1A34, *B. megaterium* 1A47, *B. cereus* 1A54, *B. cereus* 1A31, *B. cereus* 1A27, and *B. subtilis* 1A52. Three media were used for the production of spores: "G" medium of Stewart and Halvorson (1953) with 2% agar and a 10-fold increase in calcium; the plating medium used by Halvorson (1957), consisting of 5 g Trypticase, 5 g yeast extract, 5 g sodium chloride, and 20 g agar per 1000 ml water, hereafter referred to as "P" medium; and tomato juice agar (Difco Laboratories, Detroit, Mich.), hereafter referred to as "T" medium. The incubation temperature used in all instances was 30 C.

Cultures grown for 24 hr in Trypticase soy broth were used as inocula. The broth inocula were spread over agar surfaces in large Pyrex baking dishes (8 by 12 by 2 in.) covered with aluminum foil. Sporulation generally was complated within 4 to 6 days and was considered adequate if 90 to 95% of the cells had produced spores. After sporulation was complete, the plates were refrigerated at 4 C for 18 to 24 hr to aid in the lysing of remaining vegetative cells.

The following procedure proved most satisfactory for washing and cleaning spores. Growth was washed from the agar surfaces with acidified distilled water (pH 2.0 to 2.5); then the spores were washed six to eight times by alternate suspension and centrifugation in acidified water. One wash in 35% dioxane was used to assist in removal of residual vegetative matter (Hardwick and Foster, 1953). After exposure to dioxane for approximately 5 min, the spores were centrifuged and given two or three more washes with distilled water. The number of washes depended upon the appearance of the supernatant and the spore pack. At this point the spores were treated at 37 C for 30 min with lysozyme (0.5 mg/ml spore suspension). The lysozyme-treated preparation was washed two or three times with alkaline distilled water (pH 10.5 to 11.0). These treatments were followed by three washes in deionized, distilled water. The cleaned spores were suspended in deionized, distilled water and stored at 4 C. During the washing procedure the spores were kept chilled. Few, if any, vegetative cells or germinated spores were present in the final preparation. None of the components for which analyses were to be made was detected in the supernatant. The presence of germinated spores and vegetative cells was determined by staining with Conklin's modification of the Wirtz' spore stain, except safranin was used as the counterstain (Manual of microbiological methods, 1957), or carbol fuchsin was the stain and methylene blue the counterstain (Powell, 1950).

Relative heat resistance of the spore preparations was determined by an adaptation of the method of El-Bisi and Ordal (1956): 200 ml of a 0.2 M phosphate buffer (pH 7.0) was heated in a constant temperature oil bath at 100 C. Sufficient spore suspension was added to obtain approximately 10^9 spores/ml. The spores were kept in suspension by constant stirring, and precautions were taken to prevent changes in volume due to evaporation. Samples were taken at 3-min intervals and chilled instantly in an ice bath. The number of surviving spores was determined by plating on "P" medium to which 0.1% soluble starch had been added.

Analyses for various spore constituents were made as follows: phosphorus by the Fiske-

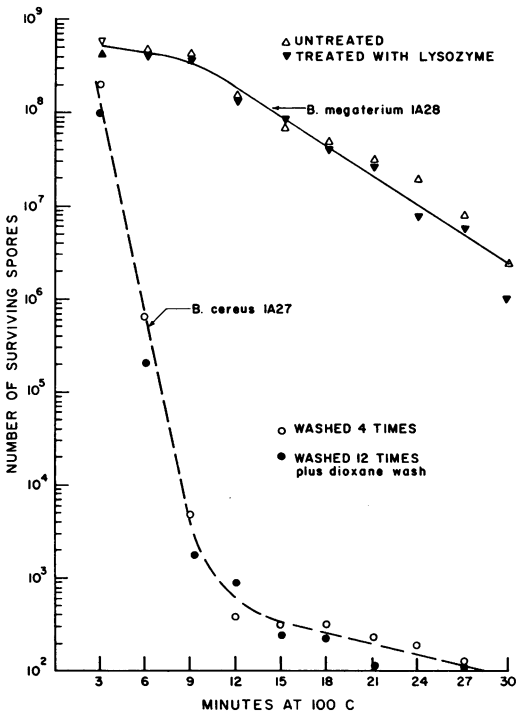

FIG. 1. *Effect of washing with acidified water, dioxane, and treatment with lysozyme on the thermal survival of spores of Bacillus megaterium 1A28 and B. cereus 1A27.*

Subbarow method; nitrogen by the micro-Kjeldahl method; carbohydrate by the anthrone method, with glucose as a standard. The procedures followed were those described by Umbreit, Burris, and Stouffer (1957). Dipicolinic acid was determined by the method of Janssen, Lund, and Anderson (1958); calcium and magnesium by an adaptation of an ethylenediaminetetraacetic acid-titrating method developed by Bird et al. (1961). For this latter determination, the spores were digested in a concentrated mixture of three parts of concentrated HNO_3 to one of H_2SO_4. This mixture was heated in a sand bath at such a temperature that the acid would condense approximately half way up the tube. Superoxol (30% H_2O_2) was added at intervals to aid in digestion. Unidentified materials were present that sometimes interfered with the end point of this titration. Such interference was avoided by filtration of the diluted and neutralized acid digest and by addition of cyanide. Recoveries of 100 ± 5% could be made upon addition of known quantities of calcium to spore digests. Analyses are reported on a dry weight basis. Dry weight was determined by drying a measured quantity of spore suspension to constant weight at 55 C.

TABLE 1. *Effect of washing and lysozyme treatment on nitrogen, phosphorus, dipicolinic acid, and carbohydrate content of spores*

Organism and Treatment	Constituents (% dry weight)			
	N	P	DPA	CHO
B. cereus IA27				
Washed 4 times	10.46	0.92	5.98	2.08
Washed 8 times	9.79	0.88	5.50	1.82
Washed 12 times plus dioxane wash	9.67	0.85	5.35	1.91
B. megaterium IA28				
Untreated	10.89	1.03	8.52	1.89
Treated with lysozyme	10.60	1.01	8.60	1.83

RESULTS AND DISCUSSION

The procedure adopted for cleaning spores was rigorous; every attempt was made to assure removal of materials other than spore constituents. In preliminary experiments, determinations were made to measure the extent to which the cleaning procedure affected the chemical composition and heat resistance of spores.

TABLE 2. *Nitrogen, carbohydrate, phosphorus, calcium, magnesium, and dipicolinic acid in spores of Bacillus spp.*

Organism	Strain	Growth medium	% Dry weight					
			N	CHO	P	Ca	Mg	DPA
1. *B. megaterium*	1A28	T	10.60	1.80	1.01	2.9	0.2	8.6
B. megaterium	1A34	G	9.23	1.96	0.76	3.7	0.8	11.1
B. megaterium	1A28	G	13.58	2.40	1.28	2.4	0.4	7.7
B. polymyxa	1A39	P	10.61	4.15	0.88	2.4	0.4	7.7
B. megaterium	1A28	P	11.26	1.30	0.78	2.4	0.7	5.6
B. cereus	1A54	T	13.36	2.58	1.08	2.6	0.4	8.3
B. polymyxa	1A39	G	11.82	4.66	0.53	1.8	0.3	6.8
2. *B. megaterium*	1A47	T	9.36	2.20	0.92	2.1	0.6	5.3
B. megaterium	1A47	T	13.40	2.87	1.11	1.6	2.2	8.7
B. cereus	1A27	P	8.69	1.30	0.74	2.1	1.2	4.4
B. megaterium	1A28	G	13.86	4.01	1.40	3.1	1.0	8.0
B. cereus	1A31	T	9.70	2.37	1.12	2.3	1.3	8.7
B. subtilis	1A52	T	11.86	3.80	1.01	1.6	1.0	7.8
B. subtilis	1A52	P	12.30	1.54	1.05	1.8	0.7	8.0
3. *B. megaterium*	1A34	T	9.35	2.60	1.20	2.7	0.3	5.7
B. polymyxa	1A39	T	13.72	—	—	2.4	0.5	10.8
B. subtilis	1A52	T	9.04	1.46	1.03	2.7	0.7	8.2

Figure 1 shows that the thermal effect of the number of washes or a dioxane wash on spores of *B. cereus* 1A27, and the exposure of spores of *B. megaterium* 1A28 to lysozyme for 18 hr at 37 C, was negligible. In this instance, the treatment with lysozyme was extended excessively to exaggerate any possible changes that might occur; the usual length of time for treatment was 30 min.

With continued washing, some changes in certain constituents were observed (Table 1). Before treatment with lysozyme, the greatest decreases were observed for nitrogen and DPA, with slight decreases in carbohydrate and phosphorus content. Excess phosphorus and carbohydrate, as well as other constituents, were probably removed in the initial four washes. These decreases could be attributed to the removal of vegetative debris and media components. Lysozyme treatment caused little change in the chemical constituents, except that the nitrogen content decreased. In stains of some of the preparations, material that stained similar to vegetative cells was evident, but it disappeared after treatment with lysozyme. The values for the chemical analyses remained relatively constant after treatment with lysozyme.

In Table 2, the batches of spores have been listed, as nearly as can be judged, in the order of their observed heat resistances (*viz.* Fig. 2 and 3) with the analyses for different constituents. Since differences in heat resistance between some of the batches of spores were not great, the order of listing for these was somewhat arbitrary. However, differences in resistance between groups were more clearly defined. Those organisms included in group I were considered to have the greatest resistance to heat, while those in groups II and III had the least resistance, based on the ability to survive at 100 C.

The values for nitrogen content, 8.5 to 13.5% on a dry weight basis, were similar to those previously reported in the literature (Murrell, 1955). According to Tinelli (1955), vegetative cells of *B. megaterium* contain 6.2 to 6.5%

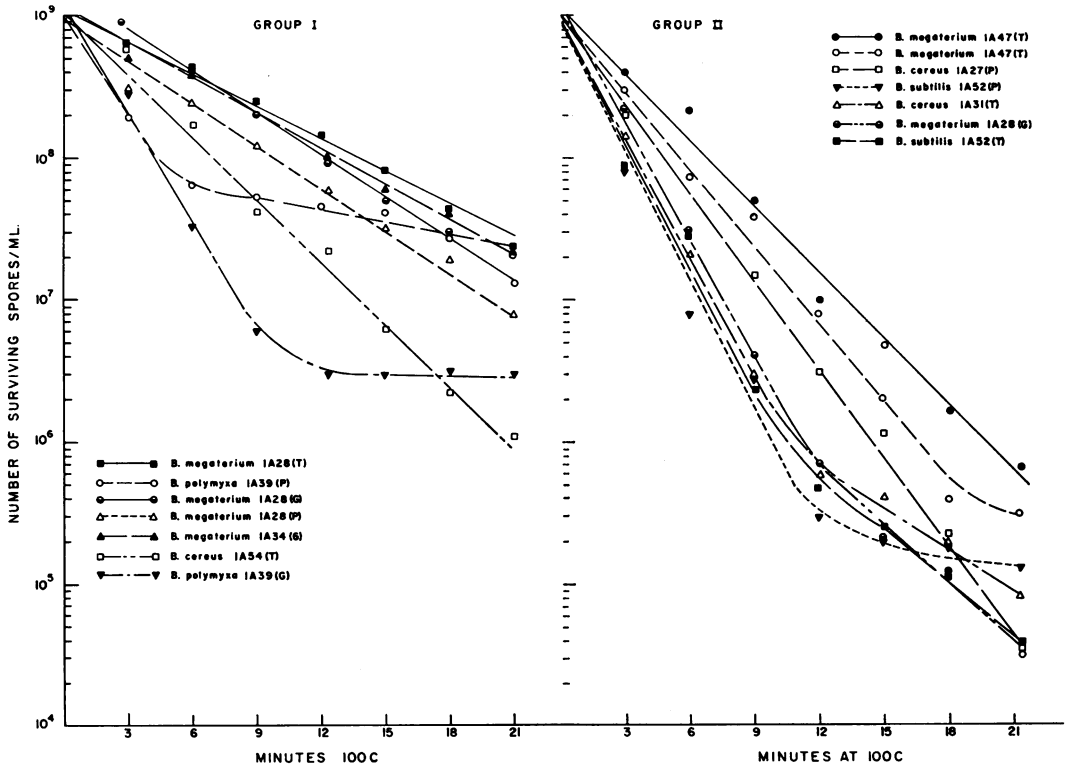

FIG. 2. *Thermal survival curves of spores of Bacillus species. Letters in parentheses indicate medium upon which spores were produced. See text for explanation.*

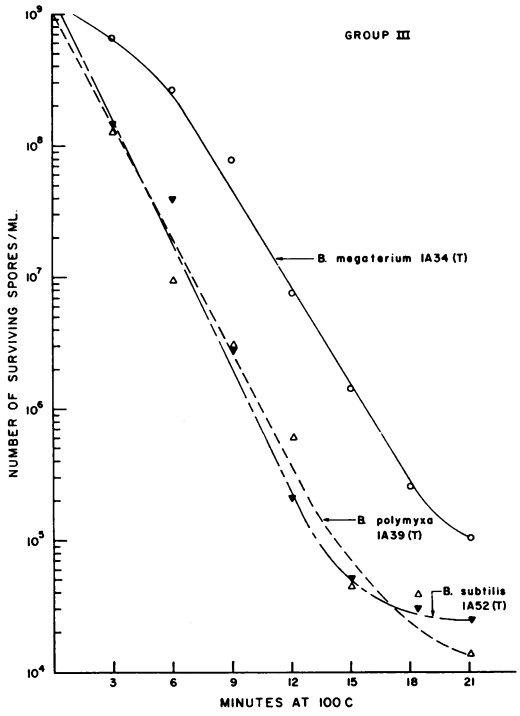

FIG. 3. *Thermal survival curves of spores of aerobic bacilli which did not fit into groups I and II on basis of survival and chemical analyses.*

nitrogen on a dry weight basis, while spores contain 11 to 12%. Tarr (1933), however, could show little difference between nitrogen content of vegetative cells and spores of *B. subtilis*. In some thermophilic bacilli, the total nitrogen appeared to be slightly higher, ranging from 12.6 to 14.2% (Sobotka and Luisada-Opper, 1957). Although considerable variation was observed in nitrogen content (Table 2), no quantitative relationship with heat resistance could be detected.

The total carbohydrate content, as estimated by the anthrone method, varied between 1.3 and 4.7% (as glucose) for the different crops of spores, with no apparent relationship to the resistance of the spores (Table 2). Tinelli (1955) found approximately 4 to 5% polysaccharide in spores of *B. megaterium* and 18 to 19% in vegetative cells. It must be recognized that the anthrone method is a means of making a gross analysis and does not differentiate the types of carbohydrate that might be present.

In most instances, the phosphorus content was higher in the groups of spores considered to be least heat resistant (groups II and III) than in the group of highest resistance (group I); however, there were several notable exceptions to this trend (Table 2). These phosphorus values

TABLE 3. *Relationship among dipicolinic acid, calcium, and magnesium contents of spores of Bacillus spp.*

Organism	Strain	Growth medium	mMoles/100 mg spores			Ratio		
			DPA	Ca	Mg	DPA/Ca	DPA/Mg	Ca/Mg
1. *B. megaterium*	1A28	T	0.060	0.073	0.008	0.8	7.5	9.1
B. megaterium	1A34	G	0.078	0.093	0.033	0.8	2.4	2.8
B. megaterium	1A28	G	0.054	0.060	0.017	0.9	3.2	3.5
B. polymyxa	1A39	P	0.054	0.060	0.017	0.9	3.2	3.5
B. megaterium	1A28	P	0.039	0.060	0.021	0.7	2.0	2.9
B. cereus	1A54	T	0.058	0.065	0.016	0.9	4.0	4.0
B. polymyxa	1A39	G	0.048	0.045	0.013	1.1	3.7	3.5
2. *B. megaterium*	1A47	T	0.037	0.052	0.025	0.7	1.5	2.1
B. megaterium	1A47	T	0.061	0.040	0.092	1.5	0.7	0.4
B. cereus	1A27	P	0.031	0.052	0.050	0.6	0.6	1.0
B. megaterium	1A28	G	0.056	0.078	0.042	0.7	1.3	1.9
B. cereus	1A31	T	0.061	0.058	0.054	1.1	1.1	1.0
B. subtilis	1A52	T	0.056	0.045	0.038	1.2	1.5	1.2
B. subtilis	1A52	P	0.055	0.040	0.042	1.4	1.4	1.0
3. *B. megaterium*	1A34	T	0.040	0.068	0.013	0.6	3.1	5.2
B. polymyxa	1A39	T	0.076	0.060	0.021	1.3	3.6	2.9
B. subtilis	1A52	T	0.057	0.068	0.028	0.8	2.0	2.3

(0.75 to 1.30%) are of the same magnitude as those reported by Curran et al. (1943). The determination of total phosphorus might not be as informative as the determination of various phosphate fractions. Perhaps correlation of heat stability with certain phosphate fractions of the spore (Fitz-James, 1955; Fitz-James, Robinow, and Bergold, 1954) would be of help in establishing the significance of this compound in the thermal resistance of spores.

An inspection of the values for DPA content (Tables 2 and 3) shows little quantitative relationship between this compound and heat resistance. Similar findings were reported by Byrne, Burton, and Koch (1960) in some preliminary experiments on the heat survival of spores of *Clostridium roseum*. Although the presence of DPA is essential for heat-resistant spores (Church and Halvorson, 1959; Hashimoto et al., 1960), the accumulation of this compound beyond a given level seems to have little influence on thermal stability.

The data presented in Tables 2 and 3 indicate that a relationship exists between DPA content, magnesium and calcium content, and heat resistance. The amount of calcium and magnesium found on a dry weight percentage basis was similar to that reported by Cohen and Wiener (1954) for *B. megaterium*. In Table 3 the ratios in which calcium, magnesium, and DPA occurred have been calculated. Those organisms showing the greatest heat resistance have less magnesium in relation to DPA than do the organisms with less heat resistance. In those spores showing the highest heat resistance (group 1), there was usually two to four times as much DPA as magnesium on a molar basis, while the less resistant spores (group II) had about equimolar proportions of DPA and magnesium. DPA and calcium occurred in approximately equimolar concentrations in all samples. Several authors (Powell, 1957; Young, 1959; Sugiyama, 1951) have suggested that calcium and DPA form a complex with protein which stabilizes the protein. If this is true, the data presented here indicate that an accumulation of magnesium in the spore interferes with the formation of the stabilizing DPA-calcium complex. Similarly, Slepecky and Foster (1959) reported reduced heat tolerance of spores of *B. megaterium* when zinc, manganese, or nickel were accumulated at the expense of calcium content.

Several notable exceptions occurred to this pattern. Group III in Tables 2 and 3 includes batches of spores which demonstrated poor heat survival but, upon analysis, had what was considered to be a favorable calcium and magnesium content for resistance (i.e., similar to group I). Apparently other factors were involved which were not considered.

Although no quantitative relationship was evident between heat resistance and carbohydrate and nitrogen content, the possibility of qualitative differences has not been eliminated. Differences have been noted between nitrogenous and carbohydrate compounds found in spores and vegetative cells. For example, when hydrolyzates of vegetative cells and of spores of *B. globibii* were analyzed by a paper chromatographic method, tyrosine and methionine were detected in the spores but not in the vegetative cells (Davis and Williams, 1952). Also, vegetative cells of *B. terminalis* have been shown to contain 3.5 times as much D-amino acid nitrogen as spores (Lawrence and Halvorson, 1954). In addition, Doak and Lamanna (1948) demonstrated carbohydrate haptene material peculiar to spores and suggested that a study of both the carbohydrate and protein composition might be fruitful in any attempt to determine the chemical basis for resistance and viability of spores. Thus, in any attempt to explain spore resistance on a chemical basis, both quantitative and qualitative factors must be considered.

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