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Studies on Poly-β-Hydroxybutyrate in Bacterial Spores* **

I. Existence of Poly-β-Hydroxybutyrate in Mature Spores of a Strain of *Bacillus cereus* and its Relation to the Acid-Fast Stainability

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SUMMARY

Effects of organic solvents were observed on the acid-fast stainability of mature spores of *Bacillus cereus* No. 2. It was found that a considerable amount of poly-β-hydroxybutyrate were extractable from the spores with chloroform, while this compound was acid-fast in stain. Removal of the polymer always resulted in the loss of acid-fast stainability of the spores, indicating that the polymer may be a chemical entity responsible for this particular stainability. An experiment was made on the distribution of the polymer in sonic disintegrates of the spores and the possible localization in the spore coats of the compound are discussed.

INTRODUCTION

One of the characteristic properties of bacterial spores is their acid-fastness in stain and some spore staining methods such as Moeller's method (1891) are believed to be based on this property. However, nothing has so far been shown on the chemical component responsible for the acid-fast stainability. During the course of our investigation on the chemical composition of mature spores of a strain of *Bacillus cereus*, it was observed that the extraction of lipids from the spores with acetone, an ether-ethanol mixture and chloroform in succession always resulted in the appearance of a mass of non-acid fast bodies in the residual spores, while the lipid fraction soluble in chloroform was acid-fast in stain. These results indicated a possible association of the chloroform-soluble material with the acid-fast stainability of the spores. This lead us to study the identity of the fraction and the relation of the material to the particular staining property.

MATERIALS AND METHODS

1. *Organism and cultural methods*

A laboratory strain of *Bacillus cereus* No. 2 from the collection of our Institute was used throughout this work. *Mature spores* were produced on a broth agar plate by culturing the organism at 35°C for a week. After incubation, the whole culture still containing a few vegetative cells was harvested, washed three times with chilled distilled water by centrifuga-

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** An outline of this work was reported at the 12th Meeting of the Japanese Bacteriological Association in the KANSAI district, (October 3, 1959).

tion and resuspended in a M/100 phosphate buffer (pH 7.0). The suspension was further incubated at 35°C with shaking until almost no vegetative cells were detectable. This aerated suspension was again washed three times with chilled distilled water by centrifugation at 3,000 rpm for 15 minutes* and the packed, washed spore preparation** free from vegetative cells finally obtained, was dried *in vacuo* ready for use. *Vegetative cells* of the organism in various growth stages were usually prepared from the dried spores by culturing the organisms in CYG medium composed of 0.5 per cent of casein hydrolysate, yeast extract and glucose (pH 7.0). The details will be described in a following section.

2. *Disintegration of spores*

A dried spore preparation was suspended in M/100 phosphate buffer (pH 7.0) and thoroughly disrupted with a sonic oscillator (KUBOTA) at 10 KC in the cold until the preparation was free from intact spores.*** To obtain an insoluble spore fraction, the whole sonicated preparation were centrifuged at 10,000 × *g* for 20 minutes in the cold. The precipitate was then treated with a crude trypsin (1 mg/ml) at 30°C, pH 7.8, for 30 minutes followed by repeating washings of the residue with distilled water. The final washed residue thus obtained was employed as an insoluble fraction of the spores.

3. *Dry-weight measurements*

The dry weight of the intact spores, vegetative cells and of insoluble residues were estimated by drying measured volumes to constant weight at 120°C.

4. *Staining methods*

Moeller's method (1891) with a slight modification**** and a simple staining by Loeffler's methylene blue were employed for staining vegetative cells, mature spores and insoluble fractions.

5. *Extraction of poly-β-hydroxybutyrate*

Unless otherwise specified, disrupted spores or vegetative cells were used as starting materials. Thus, a given weight of dried spores or vegetative cells was suspended in a M/100 phosphate buffer (pH 7.0) and disrupted thoroughly by sonic oscillation as described above. The intact sonicated preparation were then dried *in vacuo*.***** Poly-β-hydroxybutyrate was extracted from the dried material by Lemoigne's method (1940) with the following modification; two grams of the material was treated first by extracting the lipids with acetone and an ether-ethanol mixture (1 : 3, v/v) in succession at their boiling points for three hours. An exaustive extraction of the poly-β-hydroxybutyrate of the residual spores was subsequently made with chloroform. Since the final chloroform-soluble materials were found to contain a variety of alcohol-soluble and also water-soluble materials, they were thoroughly washed with hot water and methanol prior to the determination of their dry weight and the identification of the fractions.

6. *Analytical methods for the purified chloroform-soluble fraction*

Chemical analyses of the purified chloroform-soluble material were performed in collabora-

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* All the small fragments and granules, if present, originating from vegetative cells were removed by reapeating centrifugations at 3,000 rpm for 15 minutes.

** An electronmicroscopic examination showed that this spore preparation did not contain any morphological units other than mature spores. The details will be described in a later paper.

*** No germination occured during this procedure.

**** The steps in the procedure were as follows; 1) Staining with hot Ziehl's carbol-basic fuchsin. 2) Decolourization with 3% sulfuric acid. 3) Contrast staining with Loeffler's methylene blue.

***** For convenience, the following method of drying was sometimes used; heating at 80°C for 30 minutes followed by storage at 45° for 20 hours. No difference was obtainable by the two methods of drying.

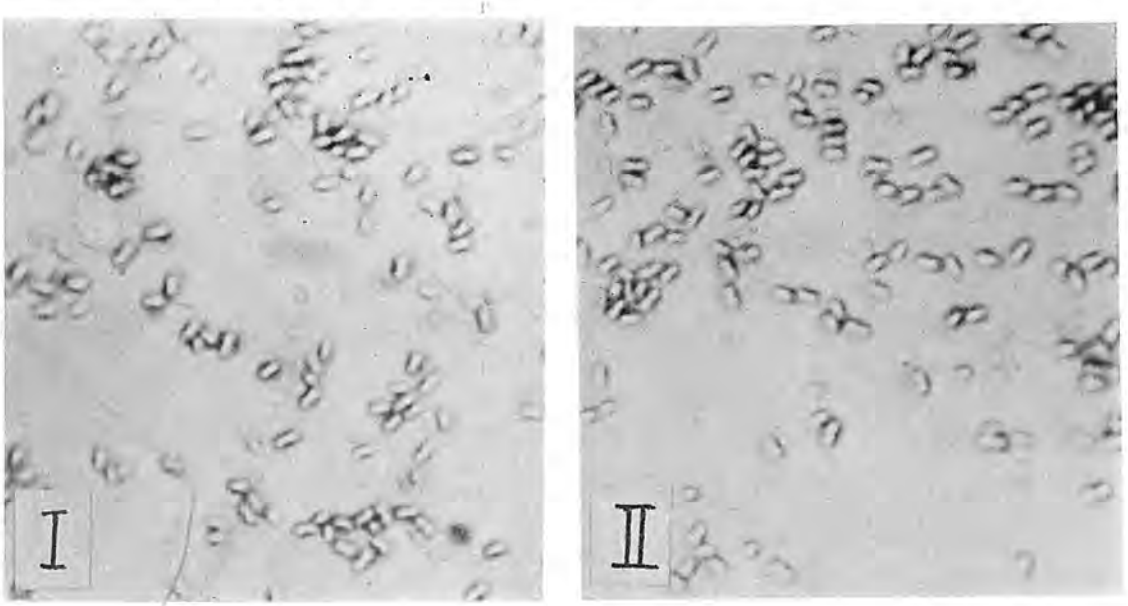
tion with Dr. K. Saito in Kobe Medical College. An elementary analysis of the material was done by the standard combustion method. Brown's method (1950) modified by Dr. Saito was employed for the paper chromatographical analysis of an alkaline hydrolysate of the material obtained after boiling for several hours with 0.5 N methanol-KOH. One dimentional paper chromatography was performed by two runs method on 50 micrograms of test samples using *n*-buthanol saturated with 1.5 N NH_4OH as solvent and a 0.4 per cent solution of brom thymol blue, as colour reagent. Thus, the sample placed on a paper chromatogram was run by a conventional ascending method and, after removal of the solvent, chromatography was repeated again using the same solvent. Authentic β-hydroxybutyric and crotonic acids were also run on the chromatogram as controls.

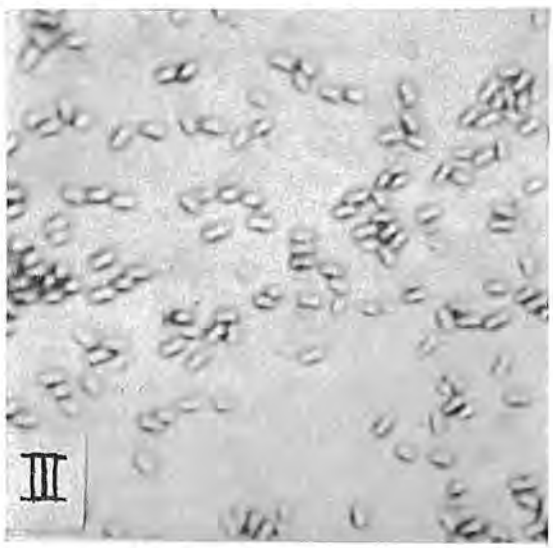
RESULTS

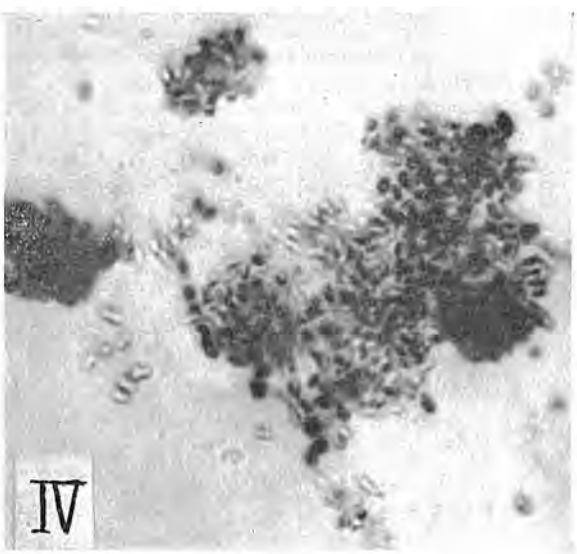
1. *Effects of organic solvents on the acid-fast stainability of mature spores of Bacillus cereus No. 2*

Dried intact spores were treated with acetone, an ether-ethanol mixture (1:3, v/v) and with chloroform in succession, and changes in the acid-fast stainability of the spores were examined after each extraction. Approximately one gram of a mature spore powder of *Bacillus cereus* No. 2 was treated first in boiling with acetone for three hours. The solvent was discarded and the spores were exposed to the ether-ethanol mixture at its boiling point for three hours. The residual spores thus obtained were further treated with boiling chloroform for three hours. Evaporation of the chloroform solution yielded a thin translucent material somewhat like a plastic film. The material was easily soluble in chloroform but not in water, acetone, ether, ethanol or methanol, and appeared to be a single component. The spores treated

Fig. 1 Changes in stainability of mature spores of *Bacillus cereus* No. 2 during the treatment with organic solvents.







Photographs; I) Intact spores.
II) Acetone treated spores.
III) Spores treated with ether-ethanol mixture.
IV) Chloroform treated spores showing densely staining forms with methylene blue.
Stained by Moeller's method. Magnification × 1,260.

with these solvents were stained by a modification of Moeller's method (1891). Thus, smears of the spores were prepared after each extraction step, stained and examined under a light microscope. Changes in the stainability of the spores during treatment with these solvents are illustrated in the accompanying photographs.

The most significant result was obtained by chloroform-treatment. As is seen in photograph IV, a number of bodies staining densely with methylene blue (a dye used for contrast stain) appeared after the treatment of spores with chloroform. These bodies were easily stainable by methylene blue and showed no acid-fastness in stain. These non-acid fast bodies appeared to be considerably smaller than intact mature spores.

These bodies increased as more chloroform-soluble material was extracted. No such change was observable even when intact spores were vigorously treated with both acetone or the ether-ethanol mixture. Photographs II and III show the stained spores treated with acetone, and acetone and the ether-ethanol mixture respectively. It will be seen that they stain very much like untreated spores. However, the red colour of the spores due to carbol-basic fuchsin became much more intense after treatment with these organic solvents. The chloroform-soluble material was acid-fast in stain.

2. *Existence of poly-β-hydroxybutyrate in mature spores of Bacillus cereus No. 2*

The results obtained in the preceding section revealed that extraction of a chloroform-soluble material from mature spores always resulted in loss of the acid-fast stainability, indicating that the particular stainability of the spores may be closely associated with the material similar to poly-β-hydroxybutyrate isolated from vegetative cells of *Bacillus* by previous workers. Examinations were thus made of the chemical nature of the chloroform-soluble fraction. In the present section the quantitative extraction and chemical analyses of this material are described.

Two grams of a mature spore powder of *Bacillus cereus* No. 2 was treated with acetone, an ether-ethanol mixture (1:3, v/v) and with chloroform in succession by the method described in the section of *Materials and Methods*. Evaporation of the chloroform solution yielded approximately 80 milligrams of a thin translucent film-like material. Although the material appeared to be a single component, it was found still to contain a variety of water-soluble (ninhydrin reactive substances) and alcohol-soluble materials. Therefore, the material was washed exhaustively with hot water and with methanol until these substances were no longer detectable. The dry weight of the final purified material was estimated to be 81 mg. Chemical analyses were made of this material. Measurements by the micro-Kjeldahl and the Fiske & Subbarow (1925) methods showed that it contained negligible amounts of nitrogen and phosphorus. It had a melting point (166-170°C) and, on heating, the material gave off white fumes with an odour characteristic of crotonic acid. On paper chromatograms in the 2nd run of the method in the preceding section, three spots were detectable. The Rf values (0.18, 0.41) of the two major spots corresponded to those of authentic β-hydroxybutyric and crotonic acids while the spot (Rf; 0.30) in between them was unidentified. Combustion analysis gave this material an empirical formula of $(C_4H_6O_2)n$ (see Table 1). Although a minor component detectable on the paper chromatogram has not yet been identified, all these properties indicate that the chloroform-soluble material isolated from mature spores of the organism is a polymer of β-hydroxybutyric acid similar to that isolated from the vegetative cells of several species of *Bacillus* including this particular organism (Lemoigne, 1940; Akashi, 1944; Lemoigne *et al.*, 1952; Tinelli, 1955; Williamson and Wilkinson, 1958). The polymer was also directly extractable from intact mature spores of the organism either by using the modification of Lemoigne's method described above or by exhaustive treatment of the spores with boiling coloroform. However, as shown inTable 2, the efficiencies of extraction by these methods were 26 per cent or much less than the efficiency when dis-

Table 1. Analysis ot the polymer extracted from mature spores of *Bacillus cereus* No. 2

	C	H
Calculated	55.57	7.288
Found	55.80	7.03

Method: Combustion analysis.

Table 2. Polymer contents of mature spores of *Bacillus cereus* No. 2 and the efficiency of some extraction methods

Material	Polymer Content	
	% dry weight of the organism	Yield (%)
Disrupted spores	4.1	100
Intact spores	3.0	74
Intact spores**	trace	—

* Dry weight of organisms was estimated by drying measured volumes to constant weight at 120°C.
** Directly extracted in boiling chloroform.

rupted spores were employed as a starting material. Particularly, direct extraction in boiling chloroform sometimes yielded only traces of the polymer from intact mature spores.

3. *Distribution of poly-β-hydroxybutyrate in the disrupted preparation of mature spores and the stainability of an insoluble fraction*

Since considerable amounts of poly-β-hydroxybutyrate were found in mature spores of *Bacillus cereus* No. 2, the localization of the polymer in the spores was studied. Insolubility of the polymer in physiological solvents suggested that it might be localized in an insoluble fraction of disrupted spores. Furthermore, if poly-β-hydroxybutyrate is the chemical entity responsible for the acid-fast stainability of the spores, the localization of the polymer and of the acid-fast stainable fraction must be identical.

Two grams of dried intact spores were therefore disintegrated exhaustively in a sonic oscillator and then fractionated by centrifugation into two parts as described in the preceding section. The supernatant fraction and the residual precipitate were dried and then the polymer was quantitatively extracted with chloroform. The residue was rapidly dissolved in boiling chloroform. As shown in Table 2, the polymer content of this fraction was 53 per cent of the dry weight, while only traces of the polymer could be extracted from the supernatant fraction.

Toble 3. Distribution of Polymer in a spore disintegrates

	Materials	Polymer Content	
		% dry weight of* the materials	Yield (%)
Fractions obtained by centrifugation at 10,000×g for 20 minutes	Supernatant (Soluble fraction)	trace	negligible
	Insoluble residues**	53	ca 100

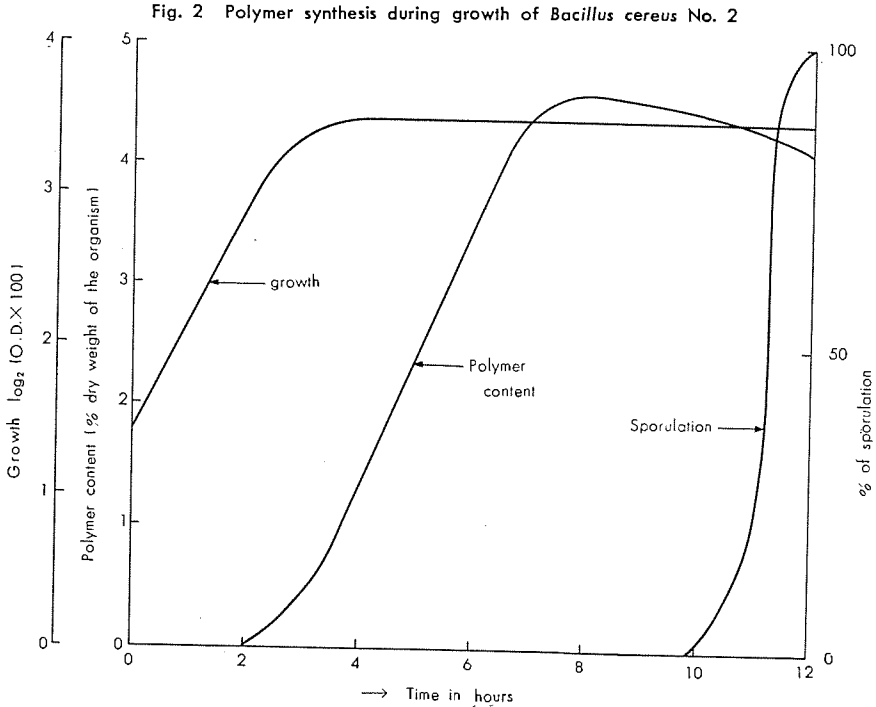
* Dry weights of organisms were estimated by drying measured volumes to constant weight at 120°C.
** Treated with 1mg/ml crude trypsin.

The polymer in the soluble fraction may be due to traces of insoluble residue which were not precipitated by centrifugation at 10,000×*g* for 20 minutes. Staining these fractions gave a rather striking result. The supernatant fraction was easily stainable by methylene blue and the stain was not acid-fast. On the contrary, a typical acid-fast stainability was recognized of the insoluble fraction. Moreover, the stainability was completely removed by extracting the polymer of the fraction with chloroform and the final dark greyish insoluble residue in the solvent stained weak purple blue with methylene blue.

4. *Kinetic observations on the synthesis of poly-β-hydroxybutyrate during growth of Bacillus cereus No. 2 and the staining of the polymer isolated from vegetative cells*

Although no reports have appeared on the polymer in bacterial spores, it has been generally admitted that vegetative cells of a variety of *Bacillus* species contain large quantities of poly-β-hydroxybutyrate. Recently, Macrae and Wilkinson (1958) has shown that the polymer is a major component of "lipid granules" isolated from the vegetative cells of *Bacillus cereus* and of *Bacillus megaterium*. The chemical nature of the polymer appears to be similar to that described in the present paper. The question therefore arose of whether these polymers are identical in terms of

Fig. 2 Polymer synthesis during growth of *Bacillus cereus* No. 2



Conditions: The organisms from 1.5 hour cultures were grown at 35 C with shaking in CYG medium. The initial pH was 7.0. The number of spores in stained smears were counted under a light microscope. The figures show the percentage of spores in stained organisms.

their acid-fast stainability. In other words, did different sources of the polymer show different staining properties ? Therefore, attempts were made to study the production of the polymer by our strain during growth and to investigate the staining of the polymers isolated from the cells at various growth phases.

To obtain vegetative cells at various growth phases, mature dried spores were inoculated into a CYG medium containing 0.5 per cent casein hydrolysate, yeast extract and glucose. The initial pH of the medium was 7.0. The dried spore powder of *Bacillus cereus* No. 2 was suspended in distilled water with Potter-Elvejhem type homogenizer and heat-shocked at 65°C for 15 minutes. A given amount of the thick spore suspension corresponding to 50 mg dry weight was then inoculated into 500 ml flasks containing 100 ml of CYG medium and incubated at 35°C with continuous shaking for 1.5 hours. After incubation, the whole culture was quickly chilled in ice and washed twice in chilled medium by centrifugation. All spores were found to have germinated. The washed packed cells thus obtained were resuspended in the medium and a given volume of the thick suspension was distributed into 500 ml flasks containing 100 ml of CYG medium. These flasks were then incubated at 35°C with continuous shaking for further 12 hours. At intervals during the incubation, aliquots were taken for measuring the growth and polymer content. Growth was determined by measuring the optical density of the culture at 550 mμ in the Coleman Junior type spectrophotometer. Polymer was extracted from vegetative cells by a method similar to that used for the mature spores. The results are illustrated in Fig. 2.

As can be seen, the organism grew exponentially for 2 hours and the growth rate under these conditions was 0.75. Growth then gradually decreased and finally ceased after 5 hour incubation. Spore formation occured very rapidly in the later stages of the stationary growth phase. A microscopic examination showed that endospores began to appear after 10 hours incubation and after a further two hours incubation, sporulation of the oragnism was almost complete. No acid-fast stainability was observable in vegetative cells regardless of their growth phase. However, the vegetative cells of the phase with a declining growth rate and particularly of the stationary growth phase stained unevenly with methylene blue, while exponentially growing cells stained uniformly.

Accumulation of poly-β-hydroxybutyrate began at the phase with a declining growth rate and reach a maximum a few hours before sporulation. The maximal polymer content of the organism was 4.6 per cent of the dry weight. No polymer could be extracted from exponentially growing cells. The stainability of the polymer isolated from vegetative cells at various growth phases were examined. All were found to be acid-fast stainable. Investigation of the production of the polymer during growth showed a reduction in content at later stages. However, the decrease was not so marked as that described by previous workers.

5. *Treatment of vegetative cells in stationary phase with acetone and an ether-ethanol mixture and changes in their staining*

Accumulation of the polymer was maximal a few hours before the appearance of endospore. At this stage of growth, the cells stained unevenly and no acid-fast

stainable structures were detectable. The polymer isolated from these cells, on the other hand, possessed a typical acid-fastness in stain. Thus the particular staining property of the compound seemed to disappear when it was present in the cytoplasm as the "lipid granules" described by Weibull (1953). Williamson and Wilkinson (1958) have recently shown the presence of 11 per cent ether-soluble lipid in the granules. Therefore, it may be that in the granules a polymer-core is covered by a layer of ether-soluble lipid. A microscopic examination was therefore made of changes in the stainability of vegetative cells before and after treatment with acetone and an ether-ethanol mixture.

Vegetative cells harvested from an 8 hour culture of *Bacillus cereus* No. 2 were treated successively with boiling acetone and the ether-ethanol mixture (1:3, v/v) for 30 minutes each. After the latter solvent had been removed, smears were prepared of the final residue, stained by Moeller's method and examined under a light microscope. Untreated cells were also stained by the same procedure. Comparison of these treated- and untreated-cells was as expected. Small pink coloured bodies— that is, acid-fast stainable— appeared in the treated-cells, which could not be seen in untreated-cells. Sometimes, a number of these small bodies adhered to the outside of the cell surface.

DISCUSSION

From the observations described in the preceding sections, it is concluded that a polymer of β-hydroxybutyric acid is present as a major chemical component of the insoluble fraction in mature spores of *Bacillus cereus* No. 2. It stains acid-fast and has the same stainability as the spores. Removal of this compound causes loss of acid-fast stainability of the spores leaving a non acid-fast stainable residue.

This compound was first recognized when Lemoigne (1927) isolated a chloroform soluble lipid from *Bacillus* "M" and subsequently identified it. The polymer has since been isolated by several workers (Lemoigne *et al.*, 1940-1950; Akashi, 1944; Tinelli, 1955; Macrae and Wilkinson, 1958) from vegetative cells of a variety of species in *Bacillus*, and seems to constitute a major constituents of the intracellular lipid inclusion bodies of the celli (Lemoigne *et al.*, 1954; Weibull, 1953; Williamson and Wilkinson, 1958). On the other hand, the existence of poly-β-hydroxybutyrate in bacterial spores had never been confirmed before our present paper. On the contrary, it was said not to exist in strains of *Bacillus megateirum* by a previous worker (Tinelli, 1955). However, this is certainly not so in the strain of *Bacillus cereus* employed in this study.* As is seen in Table 2, mature spores of the organism contain 4.1 per cent polymer per dry weight. The polymer seems not to originate from Weibull's "lipid granules", since the granules or similar structures were not detectable in the starting material obtained after repeating washings by centrifugation at 3,000 rpm for 15 minutes. Moreover, from the result of our experiments on the distribution of polymer in spore disintegrates, it is clear that poly-β-hydroxybutyrate constitutes more than half the weight of insoluble residues obtained by centrifugation. Our electronmicroscopic observations which are now being carrying on in collaboration with Dr. Fukai and Mr. Nishi, reveal that the insoluble

* We have recently shown that small amounts (approximately 0.3%) of the polymer definitely exist in mature spores of *Bacillus subtilis* subtilis and other species. The polymers also stain acid-fast.

residues are mostly spore coats including exosporia. It might therefore be that the polymer is a main component of one of the spore coats. Some properties such as its insolubility in physiological solvents and chemical inactiveness seem well suited to the polymer being a major component of the spore coat.

One of the most significant results in the present study is our finding that the polymer may be responsible for the acid-fast stainability of bacterial spores. As is well known, the resistancy to acid-decolourization represents one of the characteristic features of *Mycobacteria* and of bacterial spores. However, nothing is so far known on the chemical entity bearing this particular property. The present data however definitely show that poly-β-hydroxybutyrate may be this entity in the spores of our organism. Thus the removal of the polymer always results in the loss of the acid-fast stainability of spores and the isolated, purified polymer itself shows a typical acid-fastness in stain. Another finding that the distribution pattern of the polymer is identical with that of an acid-fast stainable fraction in spores may add a further evidence to the particular role of the polymer. In a preceding section, it was noted that the intensity of the red-colour of stained spores was greater after treating intact spores with acetone and an ether-ethanol mixture. On the other hand, the polymer can be extracted from intact spores with chloroform more easily if the spores are previously treated with these solvents. One can therefore postulate that the phenomenon may be due to the removal from the spores of an outermost, non acid-fast layer which is dissociated from the inner structure by these solvents. In other words, molecules of basic fuchsin reach the polymer fraction more easily when the covering layer is stripped off from the intact spores. However, the true explanation of this phenomenon must await further obervations using an electron-microscope.

The identification of the polymer in mature spores with that in vegetative cells still remains to be clarified in terms of their chemical structure. However, the present study clearly shows that they are identical, at least in terms of their acid-fast stainability. As mentioned above, the polymer in vegetative cells has been found to be localized in "lipid inclusion bodies" as the major component, with 11 per cent ether-soluble lipid. The polymer isolated from vegtative cells shows a typical acid-fastness in stain as described in the preceding section. Nevertheless, these inclusions are never stained by Moeller's method, while the cytoplasm stains unevenly with methylene blue. From these observation it may be concluded that the polymer of the inclusion is covered by a layer of ether-soluble lipid not stainable by methylene blue, so that the potential acid-fast stainability of the granules is veiled by the lipid. The result described in the preceding section indicates that this may be so. Thus, a successful demonstration of acid-fast stainable bodies in vegetative cells can be achieved by treating the cells with acetone and an ether-alcohol mixture, prior to staining. This is similar to the reciprocal observation by Williamson and Wilkinson (1958) that the sudanophilic nature of these inclusions disappears on removal of ether-soluble lipid from them.

The true structure of a molecule of the polymer in mature spores of our organism is still uncertain. An alkaline hydrolysate of the polymer gave three spots on a paper chromatogram. One spot between those of β-hydroxybutyric and

crotonic acids is as yet unidentified. However, considering the empirical formula given by an elementary analysis, it may be that the compound is hydroxybutyric acid. Unfortunately, it is not known whether the unknown compound is a true constituent of the polymer or only an artifact. This question may be solved by further quantitative analysis of the polymer.

The maximal polymer content of our organism was 4.6 per cent of the dry weight. This seems to be much smaller than that of reported by other workers in other strains of *Bacillus cereus* and in strains of *Bacillus megaterium*. Differences in strain and in growing conditions, particularly the pH of the medium (Macrae and Wilkinson, 1958) may partially account for the low production of the polymer by our strain. Production of the polymer during growth shows a slight decrease in the contents in later stages, suggesting that a part of the polymer may be consumed before sporulation. Since much decrease in polymer occured before sporulation, it has been suggested by pervious workers that it may function as one of main storage materials which are used eventually by the cells for spore formation. Although such an hypothesis may be valid (Macrae and Wilkinson, 1958), it is only a partial explanation of the function of the polymer. As is suggested from our present data, the polymer may function in bacterial spores in protecting the spore-cores, being a major component of one of the coats. This idea will be discussed in our forthcoming paper on the localization of the polymer in its relation to the morphological structures of bacterial spores.

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