Poly- β -hydroxybutyrate Metabolism in Washed Suspensions of *Bacillus cereus* and *Bacillus megaterium*

By R. M. MACRAE AND J. F. WILKINSON

Bacteriology Department, University of Edinburgh

SUMMARY: Poly- β -hydroxybutyrate has been previously shown to be a major component of bacterial 'lipid' granules. In the present study, the conditions under which it was formed and degraded by *Bacillus cereus* and *B. megaterium* were studied in washed suspensions. Suitable substrates for synthesis were glucose, pyruvate or β -hydroxybutyrate. Acetate, although alone unable to induce synthesis, greatly enhanced formation in presence of these substrates. Under optimal conditions, suspensions synthesized up to eight times their original content of poly- β -hydroxybutyrate in 4 hr. Formation was inhibited by high concentrations of oxygen, although no synthesis occurred anaerobically in nitrogen. The optimal concentration of oxygen was about 5%. *B. cereus* only was able to synthesize poly- β -hydroxybutyrate in an atmosphere of hydrogen. In the absence of an external carbon and energy source, degradation occurred rapidly aerobically, to carbon dioxide and water, and more slowly anaerobically to β -hydroxybutyrate and acetoacetate. The evidence that poly- β -hydroxybutyrate is a reserve carbon and energy source is discussed.

Lemoigne (1923) showed that an aerobic spore-forming bacillus, designated *Bacillus* 'M' formed quantities of β -hydroxybutyric acid in anaerobic suspensions in the absence of an external carbon and energy source. In subsequent investigations (Lemoigne, 1925) he made quantitative estimations of this acid formed and concluded that it accounted for the greater part of the acidic substances produced under the conditions of his experiments. He then demonstrated (Lemoigne, 1927) that a substance having the empirical formula $(C_4H_6O_2)_n$ could be extracted from the bacilli by chloroform and he was able to show that the material was a polymer of β -hydroxybutyric acid. Subsequently it became clear (Lemoigne, Delaporte & Croson, 1944) that there was a correlation between the amount of this polymer which could be extracted and the amount of refractile 'fatty' cytoplasmic granular material exhibited by the bacilli. Confirmation that the polymer was a major constituent of these 'lipid' granules was obtained by Weibull (1953) in his observations on the nature of the granules isolated after dissolution of the cell wall of *Bacillus megaterium* by lysozyme. Williamson & Wilkinson (1958) showed that an alkaline hypochlorite solution would liberate the granules which upon recovery and analysis were shown to consist largely of poly- β -hydroxybutyrate. A rapid quantitative method for the estimation of this substance was also described. Lemoigne, Grelet & Croson (1950) drew attention to the different amounts of poly- β -hydroxybutyrate obtained by growing *B. megaterium* on different media, and Macrae & Wilkinson (1958) showed that more of this substance was formed as the glucose concentration of the growth medium was increased; the subsequent depletion of the product during the later stages of

growth suggested a storage function. Washed suspension experiments with non-proliferating organisms have hitherto been confined to the breakdown of poly- β -hydroxybutyrate. Tinelli (1955*a, b*) correlated this degradation with gas exchange observations.

The present work was undertaken to determine the conditions under which poly- β -hydroxybutyrate is formed or broken down and to gather information with a view to deciding upon its functional significance. *Bacillus cereus* and *B. megaterium* were used and the effects of various substrates, inhibitors and gaseous environments studied.

METHODS

Organisms and cultural methods. The asporogenous *Bacillus megaterium* strain KM (Northrop, 1951) and *B. cereus* strain AC (Williamson & Wilkinson, 1958) were chosen in order to avoid complication by spore formation. They were grown at 30° in a liquid medium which contained the following substances (g.) in 100 ml. distilled water: Na_2HPO_4 , 0.6; KH_2PO_4 , 0.3; NaCl , 0.3; NH_4Cl , 0.1; Na_2SO_4 , 0.01; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.001; Casamino acids (Difco), 0.01. Glucose was added to a final concentration of 0.3% or 2.0% (w/v) for the production of organisms respectively poor and rich in poly- β -hydroxybutyrate (Macrae & Wilkinson, 1958). The cultures were grown in glass tubes (50 $\times$ 5 cm.) containing 500 ml. medium and oxygenated by passing filtered air from a small electric pump through a sintered-glass distributing tube. The bacterial inoculum was 5 ml. of an overnight culture obtained after training the organism to the medium by six previous subcultures.

Harvesting was effected towards the end of the log phase (about 18 hr.) and the organisms were washed twice in 0.85 (w/v) NaCl. All washed suspension experiments were carried out in flasks shaken in a water bath, the organisms being in 0.1 M- $\text{Na}_2\text{PO}_4/\text{KH}_2\text{PO}_4$ buffer (pH 7.2) except when stated otherwise.

Assay methods. Turbidity, dry weight, total nitrogen and poly- β -hydroxybutyrate estimations were carried out by the methods described by Williamson & Wilkinson (1958). The $\frac{1}{2}$ hypochlorite-isolated granules of *Bacillus megaterium* were found to contain a variable amount of ether-soluble lipid (between 5 and 10%); the calibration curve prepared and used for poly- β -hydroxybutyrate determination is given in Fig. 1. β -hydroxybutyrate and acetoacetate were estimated by the method of Thin & Robertson (1952).

Chromatography. Short-chain fatty acids were separated by the method of Duncan & Porteous (1953). Parallel experiments were made on the 2:4-dinitrophenylhydrazones of keto acids (Cavallini & Frontali, 1954).

Chemicals. Sodium 2-hydroxy-1-propane sulphonate was prepared by the method of Stewart & Cordts (1952).

RESULTS

The degradation of poly- β -hydroxybutyrate

Bacillus megaterium utilized its stored poly- β -hydroxybutyrate when a washed suspension of organisms made rich in poly- β -hydroxybutyrate by growth in the medium of high glucose content was shaken in presence of oxygen or anaerobically in presence of nitrogen. The results in Fig. 2 show that degradation was more rapid aerobically, causing a breakdown of 61 % poly- β -hydroxybutyrate compared with 17 % anaerobically. Comparable results were obtained with *B. cereus*.

Fig. 1

Fig. 2

Fig. 1. Calibration curve for the estimation of poly- β -hydroxybutyrate in *Bacillus megaterium*.

Fig. 2. Aerobic and anaerobic breakdown of poly- β -hydroxybutyrate in organisms. Flasks (150 ml.) contained 40 ml. washed suspension of *Bacillus megaterium* (equiv. 0.089 mg. total-N/ml.) in 0.1M-phosphate buffer (pH 7.2). Gas phase air or nitrogen.

Chromatographic analyses of the supernatant liquids of centrifuged suspensions after anaerobic or aerobic breakdown showed the presence of β -hydroxybutyric acid, acetoacetic acid and smaller amounts of acetic acid, but no other aliphatic short-chain fatty acids. Amounts of β -hydroxybutyrate and acetoacetic acid after incubation in air, nitrogen or hydrogen for 200 min. are given in Table 1. The decrease in polymer of 160 μ g./ml. exhibited in presence

Table 1. *The production of β -hydroxybutyrate and acetoacetate correlated with the decrease of poly- β -hydroxybutyrate during the endogenous metabolism of Bacillus megaterium.*

Flasks (150 ml.) contained 10 ml. washed suspension *B. megaterium* (equiv. 0.59 mg. total-N/ml.) in 0.2M-phosphate buffer (pH 7.2) Initial poly- β -hydroxybutyrate concentration = 2480 μ g./ml. Incubation time 200 min.

Gas phase	Decrease in poly- β -hydroxybutyrate (μ g./ml.)	β -Hydroxybutyrate formation (μ g./ml.)	Acetoacetate formation (μ g./ml.)
Air	580	8	45
Nitrogen	160	112	59
Hydrogen	160	116	51

of nitrogen or hydrogen represents 194 μg . β -hydroxybutyric acid/ml. if a simple hydrolysis occurs. If acetoacetate were derived from β -hydroxybutyrate by oxidation as shown by Lemoigne, Péaud-Lenoël & Croson (1949) in washed suspensions of *Bacillus megaterium*, the overall yield of these acids from poly- β -hydroxybutyrate in presence of nitrogen or hydrogen was 89 and 87 %, respectively. These figures will be too high if other cellular materials are broken down to acetoacetic or β -hydroxybutyric acids. The remainder of the polymer may be further degraded to acetic acid and other products. There was no evidence for the presence of acetone formed during anaerobic breakdown, or of any gaseous products as determined manometrically. Aerobically, insignificant quantities of β -hydroxybutyrate were produced, although a much greater amount of poly- β -hydroxybutyrate was broken down and was probably oxidized to CO_2 and water. This contention is supported by parallel measurements of oxygen uptake and poly- β -hydroxybutyrate degradation. It was found, for example, that in an 8 hr. experiment where the poly- β -hydroxybutyrate concentration fell by 49 μg ./ml. (80–81 μg .) the suspension took up 130 μl . O_2 /ml. In accordance with the equation

only 57 μl . O_2 /ml. would be required for the complete oxidation of the amount of poly- β -hydroxybutyrate dissimilated.

The rate of endogenous respiration was probably dependent on the amount of poly- β -hydroxybutyrate in the organisms. Thus *Bacillus cereus* grown in media containing either 0.3 % or 2.0 % (w/v) glucose had poly- β -hydroxybutyrate/total-N ratios of, respectively, 0.83 and 3.27 and Q_{O_2} (N) values in the absence of an external substrate of, respectively, 169 and 536.

The effect of various substrates for the synthesis of poly- β -hydroxybutyrate

A washed suspension of *Bacillus megaterium* which had been grown on a medium of low glucose content to give a small initial quantity of intracellular poly- β -hydroxybutyrate, was shaken in air at 30° with a variety of compounds, all at 0.1 M. The results (Fig. 3) show that, while there was a breakdown of poly- β -hydroxybutyrate in the control, synthesis occurred in presence of sodium pyruvate, sodium β -hydroxybutyrate and glucose; sodium acetate, although producing no net synthesis, prevented breakdown. It was further found that when sodium acetate was added to glucose, sodium pyruvate or sodium β -hydroxybutyrate, there was a considerably enhanced rate of synthesis so that the organisms more than doubled their content of poly- β -hydroxybutyrate during 4 hr. When different concentrations of sodium acetate (0.0025–0.3 M) were added to a fixed glucose concentration (0.05 M), using *Bacillus cereus* under the same conditions, the total amount of synthesis of poly- β -hydroxybutyrate was proportional to the acetate concentration up to about 0.05 M and reached a peak at about 0.1 M (Fig. 4).

The high values with glucose or pyruvate + acetate suggested that the former compounds might be producing an intermediate which reacted with acetate to give a 4-carbon compound related to β -hydroxybutyrate which then

condensed to give poly- β -hydroxybutyrate. Acetaldehyde was a possible intermediate and was tested by itself and in presence of acetate or pyruvate. The results (Table 2) showed no synthesis. In fact, acetaldehyde acted as a complete inhibitor of poly- β -hydroxybutyrate synthesis down to a concentration of 0.005M. A variety of other possible substrates were tested and are listed in Table 2. Insignificant synthesis of poly- β -hydroxybutyrate took

Fig. 3. The synthesis of poly- β -hydroxybutyrate by *Bacillus megaterium* from a variety of substrates. Flasks (150 ml.) contained 40 ml. washed suspension of *B. megaterium* (equiv. 0.138 mg. total-N/ml.) in 0.1M-phosphate buffer (pH 7.2). Gas phase was air. +--+ = no substrate; ●-● = acetate; △-△ = pyruvate; ○-○ = β -hydroxybutyrate; +-+ = glucose; ○-○ = β -hydroxybutyrate + acetate; △-△ = pyruvate + acetate; ●-● =, glucose + acetate.

place with all the substances not previously tested. An analogue of β -hydroxybutyrate, sodium 2-hydroxy-1-propane sulphonate ($\text{CH}_3\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{SO}_3\text{Na}$) was also inactive as a substrate. However, many substances, while not directly inducing synthesis of poly- β -hydroxybutyrate, partially suppressed its dissimilation. The permeability properties of the bacilli were not investigated and their inability to bring about synthesis of poly- β -hydroxybutyrate from many of the substances in Table 2 may have been due wholly or partly to their unavailability to the organisms.

It was possible that the increase in turbidity of the hypochlorite-isolated granules after incubation with these substrates in washed suspension might be due to the formation of volutin granules which Williamson & Wilkinson

Table 2. *The synthesis of poly- β -hydroxybutyrate by Bacillus megaterium from a variety of substrates*

Initial poly- β -hydroxybutyrate content = 81 μ g./0.1 mg. total-N of organism. Flasks (150 ml.) contained 40 ml. of washed suspension of *Bacillus megaterium* (equiv. 0.101 mg. total-N/ml.) in 0.1 M-phosphate buffer (pH 7.2).

Substrate	Conditions of incubation			
			+ Glucose (0.05 M)	
	2 hr.	4 hr.	2 hr.	4 hr.
Poly- β -hydroxybutyrate content of organisms (μ g./0.1 mg. total-N)				
None	26	24	36	35
Acetaldehyde	30	31	—	—
Acetaldehyde + acetate	30	31	—	—
Acetaldehyde + pyruvate	29	32	—	—
Acetate	32	30	62	87
Acetoacetate	32	31	36	33
Butyrate	28	28	36	37
Caproate	28	26	37	37
Citrate	30	22	37	36
Crotonate	28	28	36	40
Formate	28	26	37	36
Fumarate	29	29	38	36
Glycerol	32	28	34	33
2-Hydroxy-1-propane sulphonate	26	24	36	35
Propionate	31	28	33	33
Pyruvate	34	37	58	84
n-Valerate	31	30	36	36

(1958) showed were stable to the hypochlorite treatment. However, in experiments of a similar nature in which a microscopical estimation of volutin was made, there was no significant increase from the low initial value, whereas there was a considerable increase in the size of the sudanophilic 'lipid' granules. A second possibility was that the increased turbidity was due solely to an increase in the ether-soluble lipid rather than to poly- β -hydroxybutyrate, although the figures of Williamson & Wilkinson (1958) for the constancy of granule composition under a variety of growth conditions makes this unlikely. To investigate this point, parallel estimations of poly- β -hydroxybutyrate by turbidity and by chloroform extraction of the hypochlorite-isolated granules were carried out, before and after incubation with suitable substrates. A washed suspension (200 ml.) of *Bacillus megaterium* (equiv. 0.27 mg. total-N/ml.) in 0.1 M-phosphate buffer (pH 7.2) containing 0.1 M-sodium acetate and 0.05 M-glucose, was made up and an initial sample of 120 ml. taken for hypochlorite treatment under the standard conditions. The remainder was incubated aerobically at 30° for 4 hr. and then treated with hypochlorite. The turbidities were measured and the granules fractionated into ether-soluble and chloroform-soluble fractions by the method described by Williamson & Wilkinson (1958). The average result of two such experiments was as follows: poly- β -hydroxybutyrate calculated from the turbidity calibration curve, initial 102 μ g./ml., final 138 μ g./ml.; chloroform-soluble material of isolated granules,

initial 98 $\mu\text{g./ml.}$, final 189 $\mu\text{g./ml.}$; ether-soluble material of isolated granules, initial 9 $\mu\text{g./ml.}$, final 15 $\mu\text{g./ml.}$ Thus the turbidity and chloroform-extraction methods for poly- β -hydroxybutyrate gave essentially the same result and the percentage of ether-soluble material in the granules remained virtually constant.

Fig. 4

Fig. 4. The synthesis of poly- β -hydroxybutyrate by *Bacillus cereus* from glucose (0.05M) in presence of different concentrations of sodium acetate. Flasks (150 ml.) contained 40 ml. washed suspension of *B. cereus* (equiv. 0.151 mg. total-N/ml.) in 0.1M-phosphate buffer (pH 7.2). Gas phase was air.

Fig. 5

Fig. 5. The effect of pH value and nature of buffer on poly- β -hydroxybutyrate synthesis by *Bacillus megaterium*. Flasks (150 ml.) contained 40 ml. washed suspension of *B. megaterium* (equiv. 0.043 mg. total-N/ml). Gas phase was air. --- = no substrate; — = 0.1M-sodium acetate + 0.05M-glucose; ○ = K phthalate + NaOH; ● = KH₂PO₄ + NaOH; × = H₂BO₃ in KCl + NaOH; △ = KH₂PO₄ + NaOH + 0.05M-tris + HCl; ▲ = KH₂PO₄ + NaOH + 0.005M-tris + HCl.

The effect of various environmental conditions on the synthesis of poly- β -hydroxybutyrate

In all the experiments on the effect of environmental conditions on the synthesis of poly- β -hydroxybutyrate from glucose and acetate, a washed suspension containing the equivalent of *c.* 0.1 mg. total-N/ml. was set up in 150 ml. flasks and shaken in air at 30°. The concentration of glucose was 0.05M and of sodium acetate 0.1M. The buffer was 0.1M-phosphate (pH 7.2) and the experimental period 4 hr., except where otherwise stated.

The effect of pH value was studied with *Bacillus megaterium* and a variety of buffers at 0.1M final concentrations. The results (Fig. 5) show that both synthesis and breakdown of poly- β -hydroxybutyrate have a fairly sharp optimum around pH 7.5. Both borate and tris (2-amino-2-hydroxy-methyl-propane 1:3-diol) buffers, however, had an inhibitory effect, as shown by comparing the points in Fig. 5 with those of the corresponding pH value with phosphate buffer. This effect was not due to lack of phosphate since phthalate buffer (pH 6.0) gave essentially the same result as phosphate buffer (pH 6.0).

Further, when phosphate was added to tris or borate buffers, no increase in synthesis was noted. These effects of pH value on poly- β -hydroxybutyrate metabolism to some extent paralleled those of pH value on oxygen consumption which, with glucose as a substrate, gave an optimum at *c.* pH 7.0. However, although borate and tris buffers inhibited oxygen consumption, the effect was much less marked than on poly- β -hydroxybutyrate synthesis. Thus the addition of 0.05 M-boric acid, 0.05 M or 0.005 M-tris buffer (pH 7.2) to 0.1 M-phosphate buffer (pH 7.2) gave respectively 36, 31 and 8 % inhibition of oxygen consumption on glucose.

The effect of adding a source of nitrogen or magnesium was next examined with *Bacillus megaterium*. Both gave a small inhibition of poly- β -hydroxybutyrate synthesis amounting to 15 % with 0.001 M-MgCl₂, 12 % with 0.001 M-NH₄Cl and 19 % with 0.01 M-NH₄Cl. With NH₄Cl, the inhibition was probably due to the intermediates and energy derived from glucose and acetate metabolism being diverted to processes connected with growth.

Sodium 2-hydroxy-1-propane sulphonate was tested as a possible competitive inhibitor of poly- β -hydroxybutyrate synthesis. Low concentrations of the analogue slightly stimulated synthesis (9 % at 0.005 M and 3 % at 0.01 M), while higher concentrations caused a slight inhibition (6 % at 0.025 M). These effects, although small, were reproducible and occurred at concentrations which had no effect on the rate of oxygen consumption under the same conditions. 2:4-Dinitrophenol, at 2.0×10^{-4} M, gave a 40 % inhibition of synthesis although the rate of oxygen consumption was slightly stimulated (2.5 %). This effect is in accord with the action of dinitrophenol as an inhibitor of carbon assimilation by preventing the synthesis of energy-rich phosphate bonds.

The effect of the gaseous environment on poly- β -hydroxybutyrate synthesis in *Bacillus cereus* was studied. Nitrogen, hydrogen, oxygen and air were used as the gas phases (frequently changed to keep the composition constant), the nitrogen and hydrogen being passed over heated copper to remove traces of oxygen. The results in Fig. 6 show the following points.

(1) Anaerobic conditions with nitrogen prevented synthesis. Observations with pyruvate (0.1 M) and acetate (0.2 M) also showed a complete inhibition of synthesis.

(2) Greater synthesis occurred in air than in oxygen, particularly over long incubation periods. In pure oxygen the organisms began to dissimilate their poly- β -hydroxybutyrate reserves during the later stages of incubation. The experiment was repeated with a variety of proportions of oxygen and nitrogen for an experimental period of 6 hr.; the results are given in Fig. 7. The optimal concentration of oxygen for synthesis was *c.* 5 % (v/v). This sensitivity to oxygen was not annulled by adding reducing agents, such as cysteine. On the other hand, different substrates showed dissimilar degrees of sensitivity. Thus the percentage inhibition of poly- β -hydroxybutyrate synthesis in pure oxygen compared with air was measured with a variety of substrates (all at 0.1 M concentration), using *Bacillus megaterium* over a 4 hr. experimental period. Glucose and acetate gave a value of 44 %, pyruvate and acetate of 77 %, and β -hydroxybutyrate and acetate of 11 %.

(3) Hydrogen gave, surprisingly, almost as good synthesis as air. However, no evidence of the uptake of hydrogen could be obtained manometrically. In fact, there was a slight output of a gas which was not absorbed by NaOH and which might have been hydrogen ($Q_{H_2}(N)=4$). Potassium cyanide at 0.005M gave a 71% inhibition of this synthesis, an effect possibly caused by action on hydrogenase which is said, under certain conditions, to be cyanide-sensitive (e.g. Hoberman & Rittenberg, 1943). The same concentration of cyanide gave a 94% inhibition of synthesis in air.

Fig. 6

Fig. 7

Fig. 6. The effect of the gaseous environment on the synthesis by *Bacillus cereus* of poly- β -hydroxybutyrate from glucose (0.05M) and sodium acetate (0.10M). Flasks (150 ml.) contained 40 ml. washed suspension of *Bacillus cereus* (equiv. 0.150 mg. total-N/ml.) in 0.1M-phosphate buffer (pH 7.2).

Fig. 7. The effect of various proportions of oxygen and nitrogen in the gas phase on the synthesis of poly- β -hydroxybutyrate from glucose (0.05M) and sodium acetate (0.10M) by *Bacillus cereus*. Flasks (150 ml.) contained 40 ml. washed suspension of *B. cereus* (equiv. 0.094 mg. total-N/ml.) in 0.1M-phosphate buffer (pH 7.2).

(4) *Bacillus megaterium* gave essentially the same results as *B. cereus* (Figs. 6, 7), except that an atmosphere of hydrogen inhibited poly- β -hydroxybutyrate synthesis in much the same way as did nitrogen. This difference may be due to a difference in the amount of hydrogenase. However, these organisms also differ in their ability to form acid from glucose under anaerobic conditions. Measurements were made in bicarbonate, with 5% carbon dioxide in nitrogen as the gas phase and a glucose concentration of 0.1M. In these conditions the equivalent of 5 mg. dry wt. *B. cereus* formed some 2.5 μ mole acid/hr. (corrected for endogenous acid production), whereas a similar mass of *B. megaterium* did not bring about any fermentation of glucose.

Another experiment was carried out to determine the effect of CO₂ on poly- β -hydroxybutyrate metabolism. No synthesis occurred with *Bacillus megaterium* when pure CO₂ was the gas phase and with 95% oxygen + 5% CO₂ (v/v) there was no stimulation of synthesis.

DISCUSSION

Various interpretations of the functional significance of bacterial 'lipid' granules have been put forward. Lemoigne (1927) described poly- β -hydroxybutyrate as a reserve material but, after further work (Lemoigne, Péaud-Lenoël & Croson, 1950) felt unable to decide whether it represented a waste or a storage product. However, Knaysi (1945) has adhered to the conception that the granules are lipoprotein particles arising from the cytoplasmic membrane which are not utilizable and may represent an abortive attempt at binary fission. Tinelli (1955*a, b*), on the other hand, found that a major part of the material was metabolized at sporulation and deduced that the two processes were intimately connected.

Poly- β -hydroxybutyrate is an ideal storage compound in so far as it is virtually insoluble in water and can thus be accumulated in large amounts as granules inside the cells. It is only weakly ionic and hence could also act as part of a neutralization mechanism by condensation of excess acidic products produced during metabolism to form this polyester. If such a neutralization mechanism were the primary function of poly- β -hydroxybutyrate, synthesis would probably have occurred best at a low pH value, but this has not been found in the present experiments. In the media used, the pH value never fell below 7.0 and yet the organisms formed up to 40% of their dry weight of poly- β -hydroxybutyrate; further, the optimum pH value for its synthesis in washed suspension was near neutrality. It is true that the external and internal pH optima may be markedly different, but it seems probable that the primary function of poly- β -hydroxybutyrate synthesis is not a neutralization mechanism. The possibility that poly- β -hydroxybutyrate is a storage compound is, however, further substantiated by the present work. In order to prove that a substance acts as a reserve carbon and/or energy source, it is necessary to demonstrate three main facts.

(1) It should best be formed in an environment containing an excess of an external carbon and energy source. Macrae & Wilkinson (1958) have demonstrated that organisms grown in a medium deficient in the carbon and energy source had a poly- β -hydroxybutyrate content many times lower (e.g. about four-fold) than organisms grown in a medium deficient in the nitrogen source only. In the present experiments, it was found that in washed suspension, a carbon and energy source was partially assimilated into poly- β -hydroxybutyrate, an assimilation which was prevented by 2:4-dinitrophenol. Under the best conditions, the proportion of poly- β -hydroxybutyrate rose from 3 to 20% of the bacterial dry-weight in 4 hr. It is to be expected that the main products of carbon assimilation will depend upon the nature of the carbon source. Thus Dagley & Johnson (1953) showed that when *Escherichia coli* was grown on media containing different amounts of glucose and acetate, lipid was formed best on media which contained an excess of acetate, while polysaccharide was formed best on media which contained an excess of glucose. Macrae & Wilkinson (1958) found that the presence of acetate in a glucose-containing medium yielded *Bacillus megaterium* in which the proportion of

poly- β -hydroxybutyrate was about 40 % of the dry weight of organism as compared with a figure of 7 % without the addition of acetate. The present experiments confirm the importance of acetate in the assimilation of carbon into poly- β -hydroxybutyrate.

(2) The supposed carbon and/or energy source should be capable of being broken down in the absence of an external carbon and energy source. Macrae & Wilkinson (1958), confirming the results of Lemoigne, Grelet, Croson & Le Treis (1945), showed that this occurred in the later stages of growth of *Bacillus megaterium*; the present experiments show that the process also occurs in washed suspension. Aerobically, dissimilation of poly- β -hydroxybutyrate in 4 hr. from 16 to 7 % of the dry weight has been observed. Lemoigne (1925) showed that β -hydroxybutyrate was the main product of anaerobic breakdown in *Bacillus* 'M', and it was surprising to find acetoacetate as well as β -hydroxybutyrate produced anaerobically in the present work. The nature of the hydrogen acceptor which allows oxidation of the product of poly- β -hydroxybutyrate breakdown to acetoacetate is not known. No evidence of hydrogen formation was obtained manometrically. Under aerobic conditions carbon dioxide and water were the main products of endogenous breakdown, although acetoacetate was also formed in small amounts. Both the organisms used were asporogenous and these results are therefore at variance with the conclusions of Tinelli (1955*a, b*) who found that complete oxidation of poly- β -hydroxybutyrate only occurred in conjunction with sporulation, and deduced that the two processes were intimately connected. However, it may be concluded that this storage product together with polysaccharide will provide the carbon and energy required for sporulation in spore-forming organisms.

(3) The products of breakdown of the storage compound should be capable of use as a source of carbon and/or energy to prevent cell autolysis and death. If poly- β -hydroxybutyrate is broken down via β -hydroxybutyrate and acetoacetate or their derivatives as the anaerobic experiments suggest, and if acetoacetate can then be oxidatively metabolized through the coenzyme A compound of acetate to carbon dioxide and water, both carbon intermediates and energy would be produced. Certainly Lemoigne, Péaud-Lenoël & Croson (1950) showed that β -hydroxybutyrate could act as the sole source of carbon and energy for the growth of *B. megaterium*, although acetoacetate could be used only with additional carbon sources (malate, aspartate, glutamate). In confirmation of the ability of the organisms used here to utilize the products of poly- β -hydroxybutyrate breakdown, the present experiments show that the rate of endogenous respiration was higher in organisms rich in poly- β -hydroxybutyrate than in organisms poor in poly- β -hydroxybutyrate. Further, Macrae & Wilkinson (1958) showed that organisms rich in poly- β -hydroxybutyrate had a slower rate of autolysis than organisms poor in poly- β -hydroxybutyrate. It is probable, therefore, that poly- β -hydroxybutyrate acts as a reserve carbon and energy source.

The present observations also provide some clues to the pathway of synthesis of poly- β -hydroxybutyrate, although interpretation of such washed suspension experiments is hampered by the undoubted interference of perme-

ability factors. The stimulatory effect of acetate on synthesis from pyruvate, glucose or β -hydroxybutyrate might be explained in two ways. (a) Acetate combines with another two-carbon compound produced during the metabolism of the other substrates. This results in the production of β -hydroxybutyrate or a derivative of it (possibly via acetoacetate) which is then polymerized. Possibly the coenzyme A complex of β -hydroxybutyrate is the active compound. (b) Acetate, by means of a mass-action effect, alters the concentration of the intermediates in either the synthesis or the breakdown of poly- β -hydroxybutyrate so as to increase the rate of synthesis or to decrease the rate of breakdown. Isotope and cell-free extract experiments are under way in the hope of providing an answer to this problem.

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