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The Isolation of Mutants not Accumulating Poly-β-hydroxybutyric Acid

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Summary. Five mutant strains of *Hydrogenomonas H 16* which synthesize poly-β-hydroxybutyric acid either slowly or not at all have been isolated following nitrite and NMG treatment of wild type cells. When grown on a nitrogen deficient agar medium, the colonies of PHB-free cells can be recognized by their diminished retention of the dye sudanblack B. Enrichment procedures for such mutants have been devised employing the ^{32}P-phosphate inactivation technique and sucrose gradient centrifugation. The mutants have been characterized with respect to their growth properties, respiratory control and other properties.

Poly-β-hydroxybutyric acid (PHB) is synthesized and transiently accumulated in many bacteria. Accumulation occurs when carbon and energy sources are in excess and when cell growth is impaired by a lack of other nutrients (Schlegel and Gottschalk, 1962). The present knowledge pertaining to the regulation of storage material biosynthesis would be contradicted if the accumulation of PHB were only the result of mass action and not subject to enzymatic regulation. Mutant strains of the hydrogen oxidizing bacterium *Hydrogenomonas H 16* which have either partially or completely lost the ability to synthesize PHB were isolated for the purpose of carrying out the relevant investigations. Methods to select, isolate, and recognize such mutants as well as the properties of some of these mutants are described in this paper.

Materials and Methods

Organisms and Cultivation. Hydrogenomonas H 16 was grown autotrophically in a mineral medium under an atmosphere of 70% hydrogen, 20% oxygen and 10% carbon dioxide (Schlegel *et al.*, 1961); for heterotrophic growth, sodium bicarbonate was replaced by 0.5% fructose or 0.2 to 0.5% organic acids and the media were aerated. Solid media were prepared by adding 2% agar. Bouillon-agar contained 0.8% Lab-Lemco-Broth (Oxoid).

Mutations were induced using 1-nitroso-3-nitro-1-methylguanidine (NMG). 10^9 cells were suspended in 10 ml mineral solution and incubated for 30 min at

30° C on the shaker; after the addition of 1 ml of a solution of NMG (2 mg/ml) the suspension was incubated for 15 min at room temperature. The cells were harvested by centrifugation, washed with mineral solution, centrifugated again, and then resuspended in 10 ml mineral solution. Aliquots of 2 ml were each pipetted into ten 50 ml Erlenmeyer flasks to which 4 ml of fructose medium were added. The total number of cells was determined in each flask following a 48 hour growth period. The suspensions were then diluted to 10^3 cells/ml and 0.1 ml from each flask plated on nutrient agar.

Recognition of PHB-Poor Colonies on Agar Plates. Colonies grown on nutrient agar plates were transferred by replica plating to a nitrogen-poor agar (0.005% ammonium chloride, 0.5% fructose). After 5 days incubation at 28° C the colonies were stained with sudanblack B (0.02% in 96% ethanol). The dye was removed after a 20 min period, and the plates were then treated for 1 min with 10 ml of 96% ethanol. Colonies containing PHB-rich cells retained the dye and appeared dark blue; colonies of PHB-deficient cells appeared light gray having lost the dye during the differentiation process.

In order to isolate mutants, colourless colonies were picked from the master plates, plated out once more and again treated with sudanblack.

One PHB-negative mutant has been isolated following enrichment by using the ^{32}P-phosphate inactivation technique (Fuerst and Stent, 1956; Harold and Harold, 1963). After treatment with nitrite and two days growth under autotrophic conditions, the cells were centrifuged, transferred to a nitrogen-free mineral medium and further incubated autotrophically for 28 hours. Under these conditions the wild type cells accumulated PHB (50% of dry weight). 1 mC ^{32}P-phosphate (0.5 ml in 0.1 N hydrochloric acid) was then added to 30 ml of this suspension (5×10^8 cells/ml). The Erlenmeyer flask was filled with a CO_2-free hydrogen-oxygen mixture and the suspension was incubated for 24 hours with magnetic stirring. The suspension was then distributed in 10 centrifuge tubes and washed with phosphate buffer. The sediment was resuspended in a mineral solution and frozen (— 20° C). The cells had incorporated 0.1 mC ^{32}P-phosphate. With the exception of the first sample examined after three days, all others were taken at intervalls of one week. After thawing each sample was streaked on agar plates. During a period of several weeks most of the cells lost their viability. After 15 days, the viable count had decreased to 5×10^4 and after 29 days to 10 cells/ml. The mutant PHB$^-$5 was isolated from a sample taken after 15 days.

The *protein content* of intact cells was determined using a modified Biuret method as described by Schmidt *et al.* (1963). *Glucose* was measured using the blood-sugar method with glucose oxidase and dianisidine for chromogenesis (Boehringer Biochemica Test Combinations). *Gluconate* determinations were carried out with a spectrophotometric test using gluconokinase and 6-phosphogluconate dehydrogenase as auxiliary enzymes. The reaction mixture contained 2.4 ml 0.05 M TEA-buffer pH 7.6; 0.2 ml 0.1 M magnesium chloride; 0.1 ml 18 mM NADP; 0.1 ml 15 mM ATP; 0.02 ml gluconokinase (1 mg/ml) and 0.1 ml test solution containing gluconate.

Quantitative Determination of PHB. The suspension was centrifuged, the cells were washed twice, resuspended in distilled water and lyophilized. 100 to 500 mg of the dry powder were extracted overnight with 30 ml chloroform at 30° C. Cells were removed by filtration (Selecta-filters, Schleicher & Schüll, no. 595 1/2) and washed once with 50 ml chloroform. PHB was precipitated by adding a 4-fold volume of diethylether. After remaining 14 hours in a refrigerator, the PHB was quantitatively obtained by using a sintered glass filter (Schott G-3). The precipitate was dried for 20 min under an infrared lamp and then weighed.

Manometric measurements were performed using a Warburg apparatus with stationary manometers (Model FL 166, B. Braun/Melsungen).

The progress of *growth* and the *accumulation of PHB* by the cells were followed by measuring the increase in turbidity while shaking the suspensions in Erlenmeyer flasks in a water thermostat at 30° C. Turbidity was measured with an Eppendorf photometer at a wavelength of 436 nm.

Sucrose Density Gradient Centrifugation. The linear sucrose density gradient was prepared from equal amounts (6.5 ml) of 90% and 40% sucrose solutions using a two cylinder mixing device. The cells were suspended in a 40% sucrose solution; a sample of 0.2 ml was then layered on top of the gradient. Centrifugation was carried out at 4° C in a Spinco Model L 65 preparative centrifuge for 1 hour at 13,000 rpm.

Experiments and Results

The Isolation of Mutants Lacking Poly-β-Hydroxybutyric Acid

In order to increase the mutation rate, the cells were treated with nitrite or 1-nitroso-3-nitro-1-methyl-guanidine (NMG) as inducing agents. To achieve a further increase of the relative number of mutants in the population, enrichment procedures were applied which aimed at killing the wild type cells. The use of particular experimental procedures is based on the following logic: wild type cells of *Hydrogenomonas H 16* accumulate poly-β-hydroxybutyric acid when growing in a nitrogen deficient medium (0.005% ammonium chloride); the storage material enables the cells to grow in the absence of an exogenous carbon and energy source (Schlegel *et al.*, 1961) although growth is rather modest. Since growing cells are killed by colistine (Reh and Schlegel, 1969), attempts were made to kill those wild type cells containing storage material by incubating them in the presence of colistine. These attempts were unsuccessful since the growth rendered possible by endogenous storage material was insufficient to permit an effective inactivation by colistine.

The ^{32}P-phosphate inactivation technique (Fuerst and Stent, 1956) for enrichment of mutants proved to be the most efficient method. The utilisation of stored PHB enabled the wild type cells to grow to certain extent and to synthesize DNA from the ^{32}P-phosphate added. Most of the cells were killed by disintegration of the incorporated radioactive phosphate while the suspension was kept in the frozen state. The number of viable cells decreased within 15 days by a factor of 10^{-4}. By streaking this sample on nutrient-broth agar and then employing replica plating on a nitrogen deficient fructose agar, the mutant PHB$^-$5 was isolated.

The other four PHB-deficient and PHB-free mutants were isolated without preliminary enrichment. In order to isolate the four strains PHB$^-$1 to PHB$^-$4, the colonies from a total of 1,100 agar plates were transferred by replica plating to agar media deficient in nitrogen and subsequently examined (Table 1).

Table 1. *Accumulation of poly-β-hydroxybutyric acid during growth on different substrates. Recognition of PHB-containing colonies using sudanblack B*

Strain or mutant resp.	Carbon and energy sources				
	Fructose	Gluconate	Acetate	β-hydroxy-butyrate	$CO_2 + H_2$
H 16 (wild type)	++++	+++	+++	++++	++++
H 16 PHB$^-$1	—	—	—	—	—
H 16 PHB$^-$2	—	—	—	—	—
H 16 PHB$^-$3	++	+++	+	++++	—
H 16 PHB$^-$4	—	—	—	—	—
H 16 PHB$^-$5	—	—	—	—	—

Growth and Accumulation of PHB on Solid Nutrient Medium

The mutant strains PHB$^-$1 to PHB$^-$5 differ from the wild type in that the synthesis of PHB is less pronounced. After four days growth at 30°C on a nitrogen deficient fructose agar, the colonies of the mutants can be differentiated from those of the wild type by staining with sudanblack B: The wild type colonies appear dark blue while the mutant colonies are either light blue or white. The results of this PHB-test are independent of the carbon source added (carbon dioxide, fructose, gluconate, acetate, β-hydroxybutyrate); this indicates that the pathway of PHB-synthesis in these mutants is blocked subsequent to the synthesis of β-hydroxybutyryl coenzyme A.

The mutant PHB$^-$5, isolated after enrichment with ^{32}P-phosphate, differs from the other mutant strains in exhibiting pleiotropic effects. It grows on solid media—autotrophically as well as heterotrophically—faster than the wild type. The mutant grows rather slowly on succinate agar. However, after an incubation period of one week papillae became visible on the colonies. From these papillae a clone was isolated which grew on succinate as well as the wild type did. In comparison to the wild type the mutant is able to grow on glucose, although rather slowly. Furthermore, it was observed that on a glucose agar medium, wild type cells in the vicinity of mutant cells were being fed by colonies of the latter. The mutant oxidizes glucose to gluconic acid which is an excellent substrate for the wild type cells.

Gluconic acid is also formed from glucose when the mutant cells are grown in a complex medium containing glucose or when washed mutant cells are incubated in a glucose containing phosphate buffer. Gluconic acid was identified chromatographically and with the aid of a combined optical test utilizing gluconokinase and 6-phosphogluconate dehydrogenase as auxiliary enzymes.

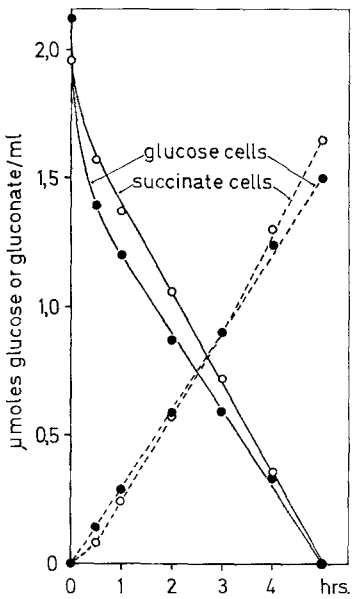

Fig. 1

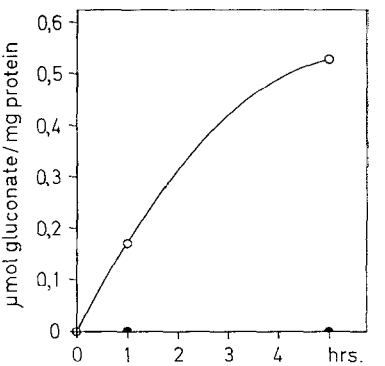

Fig. 2

Fig. 1. Oxidation of glucose to gluconate by suspensions of intact cells of mutant PHB⁻4. Succinate cells had been grown on succinate and had never had contact with glucose. The glucose cells had been grown in a succinate (0.5%) medium and then incubated for 24 hours in a medium containing 0.5% glucose. Both cell types were washed and shaken at 30° C in a nitrogen-free mineral solution containing 5 mM glucose (0.25 mg cell protein/ml). Glucose (solid lines) and gluconate (broken lines) were determined enzymatically (see Methods)

Fig. 2. Oxidation of glucose to gluconate by the particle fraction and the particle-free extract of the mutant PHB⁻4 and of the wild type. Both strains had been grown on succinate and were homogenized by sonification. The cell debris was removed by centrifugation at 7,500 g (30 min) and the particle fraction at 80,000 g (45 min). The particle fraction was resuspended in a bovine serum albumin solution (10 mg/ml). 1 mg glucose/ml was added to the particle-free supernatant (10 ml protein/ml) and to the particle suspension. The samples were shaken for 5 hours at 30° C. Gluconic acid was determined enzymatically. The formation of gluconate was catalyzed only by the particle fraction from the mutants (open circles) and not by the supernatant (full circles). The wild type fractions were completely inactive

The oxidation of glucose to gluconic acid is catalyzed by a washed suspension of intact mutant cells (PHB⁻5) at a relatively high rate of 1.6 µmoles glucose/hour/mg cell protein. The transformation is almost quantitative; the loss of glucose due to conversion to other products is negligibly small (Fig. 1). The oxidation rate was not dependent on the presence of glucose in the growth medium. Cells grown only on succinate

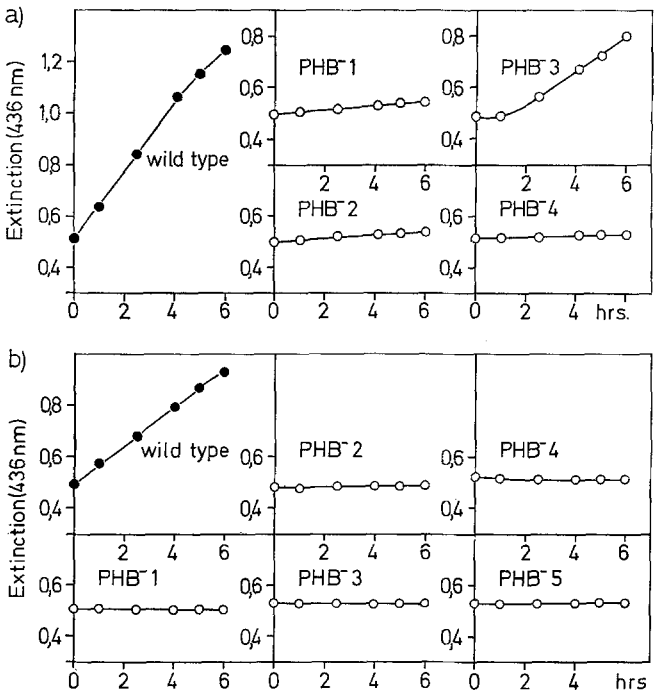

Fig. 3. Turbidity increase of suspensions of the wild type and the mutant cells during incubation in a nitrogen-free mineral solution containing a) gluconate and b) acetate as substrates under aerobic conditions

were just as active as cells first cultivated with succinate and then with glucose for 24 hours. A filtrate from the suspension did not catalyze the oxidation of glucose.

The particle fraction of the cells is responsible for the oxidation of glucose. Succinate grown cells were disrupted by sonification, cell debris removed by centrifugation at 7,500 g (30 min) and the particle fraction recovered at 80,000 g (45 min). Only the particle fraction catalyzed the oxidation of glucose to gluconic acid (Fig. 2); the particle-free supernatant was inactive. Neither the particle fraction nor the supernatant from wild type cells grown on succinate or fructose possessed glucose oxidase activity.

Growth and PHB-Accumulation in Liquid Media

Mutant strains PHB$^-$1 to PHB$^-$4 grow in both shaken and stirred cultures as well as the wild type does. In some cases, the turbidity of the suspension increased when shaken in a nitrogen deficient gluconate or

Table 2. *PHB-content of mutants and of the wild type strain of* Hydrogenomonas *following incubation in the presence of fructose, gluconate, acetate or carbon dioxide + hydrogen in the absence of a nitrogen source*

Strain or mutant resp.	Amount of PHB (per cent of dry weight) after incubation with			
	Fructose (40 hours)	Gluconate (23 hours)	Acetate (23 hours)	$CO_2 + H_2$ (26 hours)
H 16 (wild type)	65.3	27.7	37.2	35.8
H 16 PHB$^-$1	11.9	7.3	—	5.6
H 16 PHB$^-$2	—	8.6	8.2	1.5
H 16 PHB$^-$3	—	7.3	13.9	4.3
H 16 PHB$^-$4	—	0	0	0
H 16 PHB$^-$5	—	—	0	0

The cells were grown in a complete medium containing the substrates indicated. The suspension was centrifuged and the cells were resuspended in a nitrogen-free medium containing the same substrates. After the incubation period the cells were harvested, washed, and freeze-dried. 100—500 mg of dry cell powder were used to gravimetrically determine the PHB-content.

acetate medium (Fig. 3). This observation indicated that mutants were not fully incapable of synthesizing PHB and only differ from the wild type in that the rate of PHB synthesis was diminished. Only two mutants did not produce any PHB: PHB$^-$4 and $^-$5. These conclusions were confirmed by quantitative determinations of the PHB extracted from the dried cells harvested after incubation under storage conditions (Table 2).

The mutant strain PHB$^-$5 also differs from the wild type with respect to other physiological properties. Although it grew faster on solid media than the wild type did, in liquid culture it was very sensitive and did not grow regularly from small inoculums. In manometric experiments in Warburg vessels, the cells catalyzed the hydrogen oxygen reaction for only one or two hours and then ceased to oxidize hydrogen. The mutant grew faster than the wild type on solid media containing either succinate or acetate as substrates, whereas its growth was either poor or negligible in liquid media containing glutamate or under autotrophic conditions (Fig. 4). The reason for this extraordinary sensitivity has not yet been elucidated.

Respiratory Control in PHB-Free Mutants

The rate of hydrogen oxidation by washed cells of *Hydrogenomonas H 16* in the absence of a nitrogen source is influenced by carbon dioxide. In the presence of carbon dioxide the oxidation rate is almost three times as high as in its absence (Schlegel and v. Bartha, 1961). A plausible explanation for this effect was given by the hypothesis that the electron

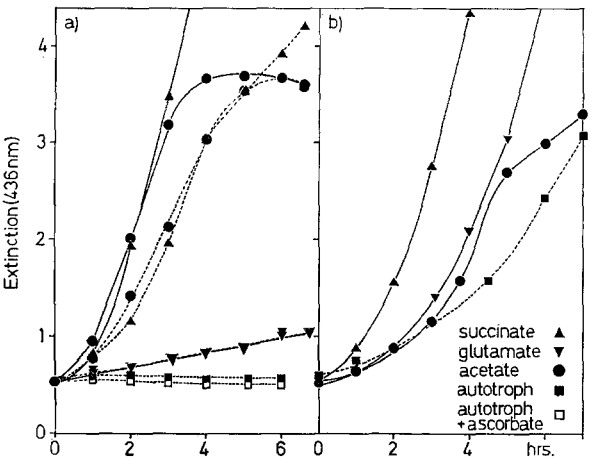

Fig. 4. Growth curves of the mutant *H 16* PHB⁻5 (a) and of the wild type (b) in media containing different substrates. The following concentrations were employed: succinate and glutamate 0.5%, acetate 0.2%, and ascorbate 0.05%. 200 ml Erlenmeyer flasks each containing 30 ml suspension were shaken under air (solid lines) and under a hydrogen oxygen mixture (80% H_2 + 10% O_2 + 10% CO_2; broken lines) in a thermostatically controlled water bath (30° C)

transport is controlled by the availability of ADP, this ADP being continually regenerated from ATP by the fixation of carbon dioxide, a process consuming very much energy. Under these conditions, carbon dioxide fixation is accompanied by the synthesis and accumulation of PHB.

Since mutants defective in PHB synthesis were now available for the first time, the confirmation of this hypothesis was made possible. If the stimulation of the electron transport in washed cells exerted by carbon dioxide was really due to ADP regeneration, cells unable to synthesize and to store PHB should exhibit no response to the addition of carbon dioxide. These cells do not fix CO_2 in the absence of a nitrogen source. The experimental results obtained under this aspect using the mutant PHB⁻4 (Fig. 5) and other PHB-free mutants were surprising. Both the mutant as well as the wild type cells catalyzed the hydrogen oxygen reaction at a constant rate. Not only did carbon dioxide stimulate the oxidation rate of the wild type cells, but also that of the mutant PHB⁻4 cells (Fig. 5). This observation is not compatible with the hypothesis outlined above and indicates a more direct effect of carbon dioxide on the electron transport process.

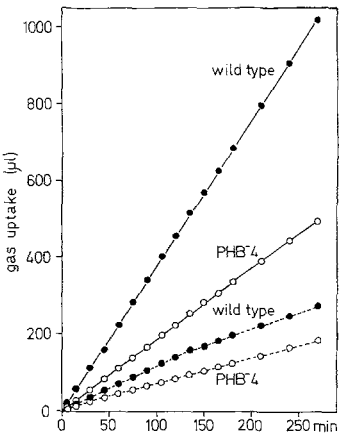

Fig. 5

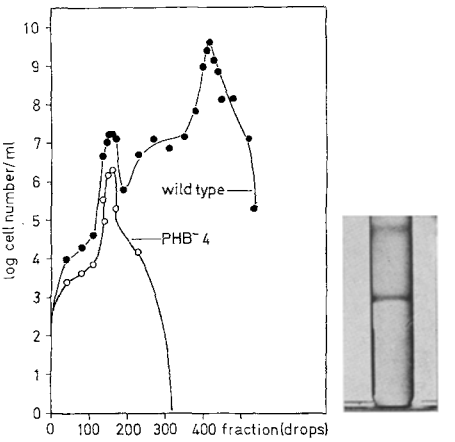

Fig. 6

Fig. 5. The rates of hydrogen oxidation by washed cells of the wild type *H 16* and the PHB-less mutant PHB⁻4 in the presence and absence of carbon dioxide. The vessels contained wild type cells (0.17 mg protein) or mutant cells (0.185 mg protein) suspended in a nitrogen-free mineral solution and were filled either with a gas mixture of 80% H_2 + 10% O_2 + 10% CO_2 or with a CO_2-free hydrogen-oxygen mixture; in the latter case the center vessel contained 0.2 ml 20% KOH

Fig. 6. The separation of PHB-containing and PHB-free cells by sucrose density gradient centrifugation. The cells of the PHB-free mutant PHB⁻4 and of the wild type of *Hydrogenomonas H 16* were mixed in a ratio of 1:25. The wild type cells contained almost 60% PHB. 0.2 ml of this mixture was layered on top of a linear sucrose density gradient (40—90%). The tubes were centrifuged for 60 min at 30,000 g, punctured, and 26 fractions of an unequal number of drops were collected. These were then diluted and plated on nitrogen deficient agar. The colonies of the mutant and of the wild type were differentiated by staining with sudanblack B and counted

Separation of PHB-Free Cells from those Containing PHB

Cells which have accumulated large amounts of PHB are characterized by having a lower specific weight than those without PHB. Therefore, the two cell types can be separated by centrifugation in a sucrose density gradient. This method appears to be a promising procedure for separating the mutant from the wild type cells.

By centrifuging a mixture of wild type cells containing 50% PHB (w/w) and the PHB-free mutants in a 40—90% linear sucrose density gradient, a separation was obtained (Fig. 6; insertion). The heavier mutant cells were located lower in the centrifuge tube and separated from the lighter wild type cells by a zone which visually appeared free of bacteria. A confirmation of the separation of the cells into the two bands was carried out by counting the bacteria. The centrifuge tube was

punctured at its base and a total of 26 fractions containing unequal numbers of drops were collected. The suspensions were diluted and plated on a nitrogen-free fructose agar. After 5 days growth, the colonies of the mutant and of the wild type were differentiated by staining with sudan-black B and then counted. The distribution of both cell types in the sucrose density gradient is depicted in Fig. 6. In principle, a satisfactory separation occurred; the mutant cells were only present in the lower band. However, the wild type cells were present in both bands.

An explanation of the fractionation diagram requires that the numerical ratio of wild type cells containing PHB to PHB-free mutants (25:1) in the original suspension be considered. This suspension (0.3 ml) had been mixed with 40% sucrose solution (0.7 ml). From this mixture, 0.2 ml were transferred to the top of the sucrose gradient. During this procedure, a considerable portion of the cells formed aggregates. During centrifugation, therefore, not only single cells but also aggregates of bacteria were separated according to their specific weights. A fraction of these aggregates were again separated into single cells during the preparation of dilution series. The first peak of the wild type distribution curve is probably due to at least three colony types: colonies from single mutant cells, colonies from single wild type cells and colonies grown from aggregates which contained both cell types. The latter colonies are identified as wild type and counted as such. Since the aggregates sedimented together with the heavy mutant cells, they must have been composed mainly of mutant cells with only a few wild type cells. When the aggregates contained a majority of wild type cells, the colonies were always identified as wild type.

This experiment offers confirmative evidence that the wild type cells and the mutant cells can be separated by density gradient centrifugation and that the PHB containing cells are characterized by a lower specific weight than the PHB-free cells. In order to separate both cell types, more effective procedures are necessary to avoid the formation of cell aggregates while suspending the cells in the sucrose solution.

Discussion

Reserve materials which are deposited intracellularly in an osmotically inert form are not indispensible cell components according to available experimental evidence. The capability to accumulate storage materials will exert a selective advantage only under those conditions in which the corresponding nutrient is lacking in the medium. Experimental proof for the ecological importance of reserve materials is still lacking (Dawes and Ribbons, 1964; Sobek *et al.*, 1966). Competition experiments between wild type strains which accumulate storage materials (poly-β-

hydroxybutyrate, starch, glycogen, and triglycerides as well as polyphosphate) and defective mutants would be very informative.

Relatively few mutants lacking reserve materials have been thus far isolated. Mutants of *Aerobacter aerogenes* which do not accumulate polyphosphates have been isolated employing the ^{32}P-phosphate inactivation technique (Harold and Harold, 1963). Mutants of *Escherichia coli K 12* (Damotte *et al.*, 1968) and *Saccharomyces cerevisiae* (Chester and Byrne, 1969) which accumulate less or no glycogen have been isolated and used for biochemical studies. A report on PHB-free mutants of *Bacillus megaterium* deals only with the isolation procedure (Bohlken, 1969).

The penicillin technique or analogous procedures can evidently not be employed for the enrichment of mutants which are unable to synthesize lipids or polysaccharides. Even after having accumulated storage materials up to their maximal cellular capacity, the wild type cells growing at the expense of their storage material are not killed in the presence of an inactivating agent. Penicillin, colistine and other antibiotics probably exert their inactivating effect only when the cells multiply several times. Only the ^{32}P-phosphate inactivation technique fulfills the requirements for a satisfactory counter selection method.

The density gradient centrifugation technique, which has been successfully employed for the separation of cell components, seems to be a promising technical method for separating cells of different specific weight. It has already been used for the separation of spores of *Bacillus cereus* lacking dipicolinic acid from wild type spores (Wise *et al.*, 1967; Halvorson and Swanson, 1969). Further experiments are necessary to avoid aggregation of the cells.

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