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Morphological Changes in Poly-β-Hydroxybutyrate Granules Associated with Decreased Susceptibility to Enzymatic Hydrolysis

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ABSTRACT

MERRICK, J. M. (State University of New York, Buffalo), D. G. LUNDGREN, AND R. M. PFISTER. Morphological changes in poly-β-hydroxybutyrate granules associated with decreased susceptibility to enzymatic hydrolysis. J. Bacteriol. **89**:234–239. 1965.—A complex enzyme system obtained from extracts of *Rhodospirillum rubrum* cells hydrolyzes poly-β-hydroxybutyric acid (PHB) contained in native PHB granules isolated from *Bacillus megaterium*. A labile factor associated with the granules and necessary for depolymerization is easily destroyed by various chemical and physical treatments. Granules inactivated by these treatments were examined in an electron microscope. In all cases, the distinct morphological appearance of native granules was altered. Morphological changes were mainly characterized by membrane fragmentation, loss of coalescence, and surface alterations. These observations suggest that native PHB granules possess definite structural features, disruption of which results in decreased susceptibility of the polymer to enzymatic hydrolysis.

Recent studies of the metabolism of poly-β-hydroxybutyrate (PHB) have shown that the enzymatic synthesis of the polymer occurs by a condensation of D(−)-β-hydroxybutyryl coenzyme A, whereas degradation of the polymer involves a complex hydrolytic enzyme system (Merrick and Doudoroff, 1961; Merrick and Doudoroff, 1964). PHB granules isolated from *Rhodospirillum rubrum* are intimately associated with both the biosynthetic and degradative enzymes of the polymer, and granules isolated from *Bacillus megaterium* contain only the synthetic enzymes. The latter PHB granules are attacked by extracts of *R. rubrum* cells which have depleted their polymer stores. The depolymerizing enzyme system consists of a thermostable activator and a thermolabile depolymerase which act successively to cause the breakdown of PHB to β-hydroxybutyric acid and small amounts of soluble esters. Under certain conditions, the activator can be replaced by trypsin. The soluble enzyme system however, will not attack native granules isolated from *B. megaterium* that have been treated with various physical and chemical agents, nor will it attack chemically purified polymer (Merrick and Doudoroff, 1964). These observations suggested that a labile component closely associated with the granules is involved in its breakdown. However, the relationship of the labile component to PHB of the granules and the mechanism whereby it participates in depolymerization have remained unclear. Recently, application of the carbon replica technique and electron microscopy have revealed the presence of a discrete membranelike substance surrounding the granules, and have demonstrated that native granules possess definite structural features (Lundgren, Pfister, and Merrick, 1964). In view of the structural and biochemical properties of native PHB granules, inactivated granules were examined in an electron microscope to see whether loss in biochemical activity is correlated with altered morphology.

MATERIALS AND METHODS

The general methods for isolation of native PHB granules from *B. megaterium* (strain KM), for preparation of the soluble depolymerizing enzyme system from *R. rubrum* (strain 111, C. B. Van Niel), and for the assay system for measuring breakdown of PHB, were previously described (Merrick and Doudoroff, 1964). Treatment of the granules by the various agents described in the text was carried out at room temperature unless indicated otherwise. PHB granules inactivated by the various treatments are no longer suitable as a substrate for the depolymerizing enzymes, and

the per cent inactivation is expressed in terms of the extent (rather than in rate) of hydrolysis of a treated preparation, compared with untreated PHB granules.

Specimens for electron microscopy were prepared by the carbon replica technique of Bradley and Williams (1957). Electron micrographs were taken with an RCA EMU-2D electron microscope; either a 50- or a 25-μ objective aperture was used.

RESULTS

Native PHB granules isolated from *B. megaterium* are shown in Fig. 1. The features to be noted are the smooth surface appearance and coalescence of the granules. Although these properties may reflect the preparative procedures for both granule isolation and electron microscopic analysis, these features are less readily seen in granules without intact membrane covers. PHB contained in such granules is susceptible to hydrolysis by the soluble enzyme system prepared from polymer-depleted cells of *R. rubrum*.

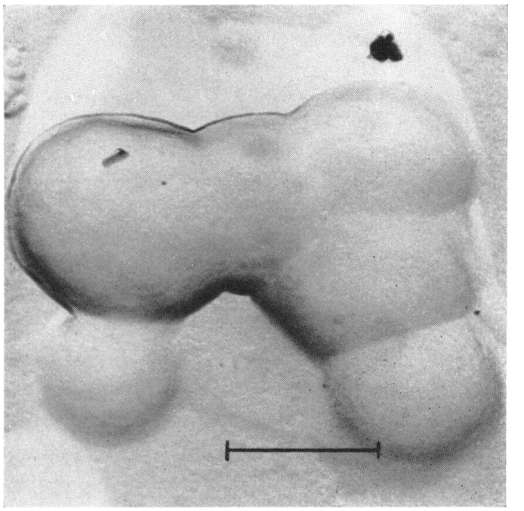

FIG. 1. *Native granules, 22,000×. The scale shown in all electron micrographs represents 1 μ.*

The effects of the various inactivating agents on native PHB granules are shown in Fig. 2 and 4. In most cases, the observed changes are loss of coalescence (with the formation of discrete granules) and membrane fragmentation.

Acetone treatment is more drastic to the granules than alcohol and the other disrupting agents under the conditions of our treatments. Acetone-treated granules (Fig. 2a) appear to have lost their complete morphological integrity, and such preparations are somewhat amorphous in appearance. The loose membranes still adhering to the granules are apparent. Alcohol-treated granules (Fig. 2b) show less of the surface membrane, but many of the granules are still largely intact and are similar to those granules inactivated by repeated centrifugation (Fig. 2d) or freezing and thawing (Fig. 2e). Alkaline hypochlorite, previously used during the isolation procedures of the polymer because it is an effective reagent in the solubilization of other cellular material (Williamson and Wilkinson, 1958), also destroys the labile component of native granules. Granules which have been inactivated by alkaline hypochlorite are seen in Fig. 2c. The membrane is partially removed, exposing the entrapped granules which still possess smooth surfaces and are morphologically intact. When native PHB granules are heated, they also become inert to the depolymerizing enzymes, and show morphological changes associated with membrane breakage; in addition, some structural detail of the polymer packed into the granule much like a ball of string (Fig. 2f) is revealed. Dramatic changes are also observed when sodium lauryl sulfate is added to native granules. Different degrees of disruption were noted, and frequently the common "lath"-shape crystals (Alper et al., 1963; Lundgren et al., 1964) making up the granules were seen (Fig. 2g; crystals are marked by arrows).

It was of particular interest to examine the effects of trypsin on PHB granules, because this enzyme both activates and inactivates the granules. Mild treatment of the granules with trypsin simulates the action of the activator fraction. Thus, PHB in trypsin-activated granules is susceptible to breakdown on the subsequent addition of depolymerase. Drastic treatment with trypsin, however, inactivates the granules, and PHB becomes resistant to depolymerization by depolymerase. The activation and inactivation effects are dependent on both the concentration of trypsin and the time of incubation. When the granules are incubated for short periods of time with low concentrations of trypsin, inactivation is not observed. The addition of salt, such as $CaCl_2$, markedly increases the rate of inactivation.

Granules activated by activator and by trypsin are shown in Fig. 3a and b. Granules treated with activator do not appear to undergo any observable morphological changes, whereas the mild trypsin treatment partially digests the membrane, leaving granules which are more discrete (loss of coalescence) and which still possess smooth surfaces. Other differences between the two types of activated granules have previously been noted (Merrick and Doudoroff, 1964).

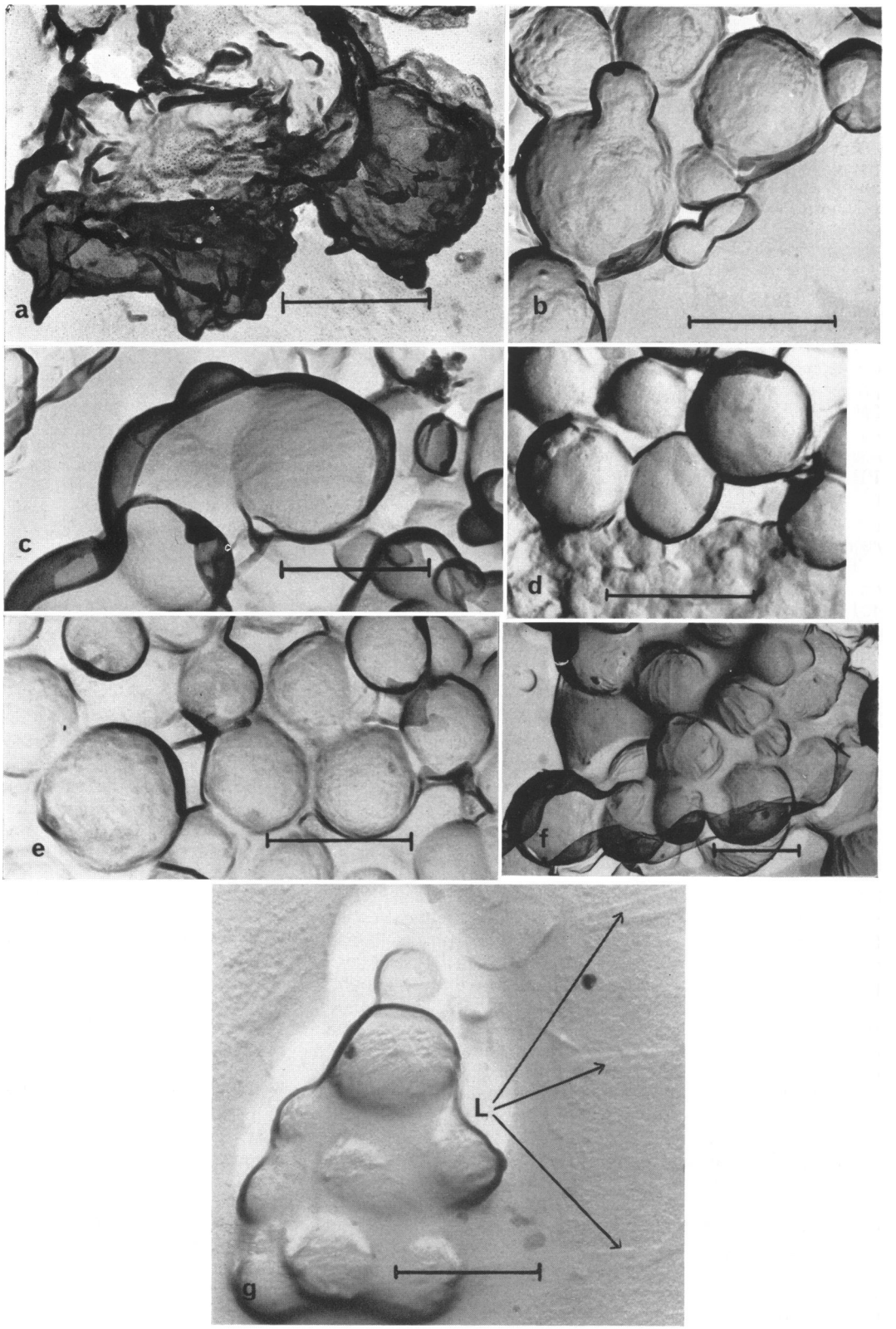

Fig. 2.

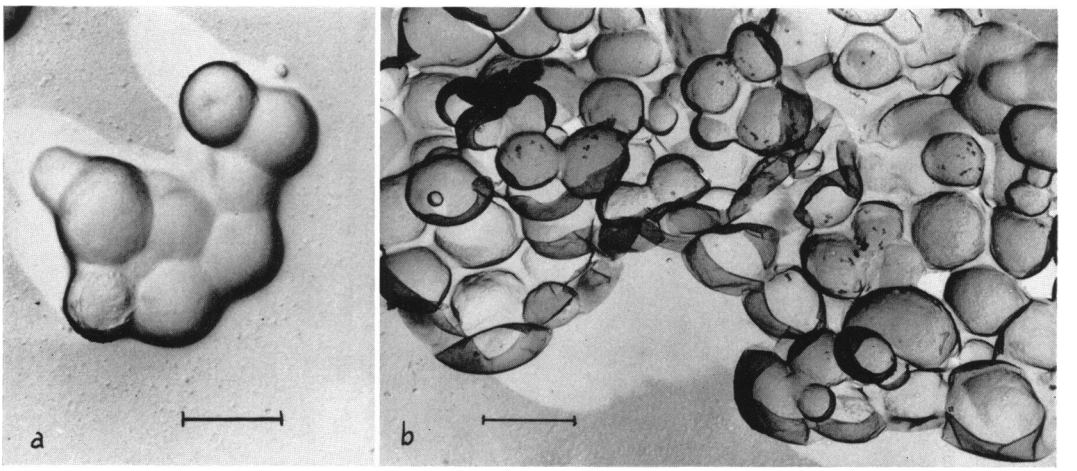

FIG. 3. *Native PHB granules (a) activated by activator, 16,500×, and (b) activated by trypsin, 15,000×. Granules were activated in the following manner: (a) the reaction mixture contained PHB granules (11 mg), activator (0.37 mg), $CaCl_2$ (1 μmole), and Tris-HCl buffer, pH 8.0 (20 μmoles), in a final volume of 1.0 ml; (b) the reaction mixture contained PHB granules (11 mg), trypsin (50 μg), and Tris-HCl buffer, pH 8.0 (30 μmoles) in a final volume of 1.0 ml. Incubations were carried out at 33 C for 30 min (a) and 15 min (b). Further action of trypsin was inhibited by the addition of ovomucoid (200 μg). Activation was followed by removing samples and determining the rate of depolymerization of PHB after addition of depolymerase (Merrick and Doudoroff, 1964). Incubations were carried out until maximal activation of the granules was obtained (that is, no further increase in rate of depolymerization was observed after the indicated time periods of activation).*

Native granules subjected to treatment with trypsin under conditions which result in inactivation of the labile component show the surface alterations seen in Fig. 4a to d. Thus, the smooth surface becomes rough and irregular with what appears to be a blistering phenomenon. This effect is approximately correlated with the degree of inactivaton of the granules.

DISCUSSION

In the present studies, native PHB granules inactivated by a variety of agents were examined in an electron microscope to relate loss of biochemical activity with altered morphology. The results show that the morphological changes vary with the different treatments. Some of the treatments result in complete loss of morphology (acetone treatment), but others appear only to involve the membrane (alkaline hypochlorite, freezing and thawing, centrifugation). Although membrane fragmentation is characteristic of all inactivated granules, its contribution, if any, to the enzymatic breakdown of the polymer is not known. Evidence has been presented (Pfister, Lundgren and Merrick, 1964; Boatman, 1964) that the granules in the cell are surrounded by a membrane, thus indicating an important metabolic relationship between the membrane and PHB. The changes observed with granules inactivated by trypsin and acetone suggest that components other than the membrane may also play a significant role in maintaining the morphological integrity of the granule.

Inactivated granules, in all cases examined, no longer possess the distinct morphological features of native granules. Morphological changes were

FIG. 2. *Inactivation of native PHB granules by various treatments (a, b, c, d, e, and g, 25,000×; f, 15,000×). Preparations of PHB granules were inactivated by suspending the granules in (a) acetone (85%) for 40 min; (b) alcohol (80%) for 40 min, and (c) alkaline hypochlorite (Williamson and Wilkinson, 1958) for 60 min at 33 C. After treatment, the inactivating agents were removed by dialysis for 24 hr against 0.02 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 8.0). Preparations d to g were inactivated by (d) repeated centrifugation (six times) at 12,000 × g for 10 min at 0 C; (e) repeated freezing and thawing (six times); (f) heating PHB granules suspended in 0.06 M Tris-HCl buffer (pH 8.0) for 5 min at 100 C; (g) sodium lauryl sulfate, 1.7% for 20 min at 0 C. Sodium lauryl sulfate was removed by centrifugation of the granules on glycerol as previously described (Merrick and Doudoroff, 1964). Such preparations become fibrous, forming "paper-like" particles which are difficult to resuspend. The per cent inactivation of the variously treated preparations is: (a) 100, (b) 100, (c) 84, (d) 88, (e) 73, (f) 86, (g) 100. In untreated granules, 85% of the PHB is hydrolyzable by the depolymerizing enzymes.*

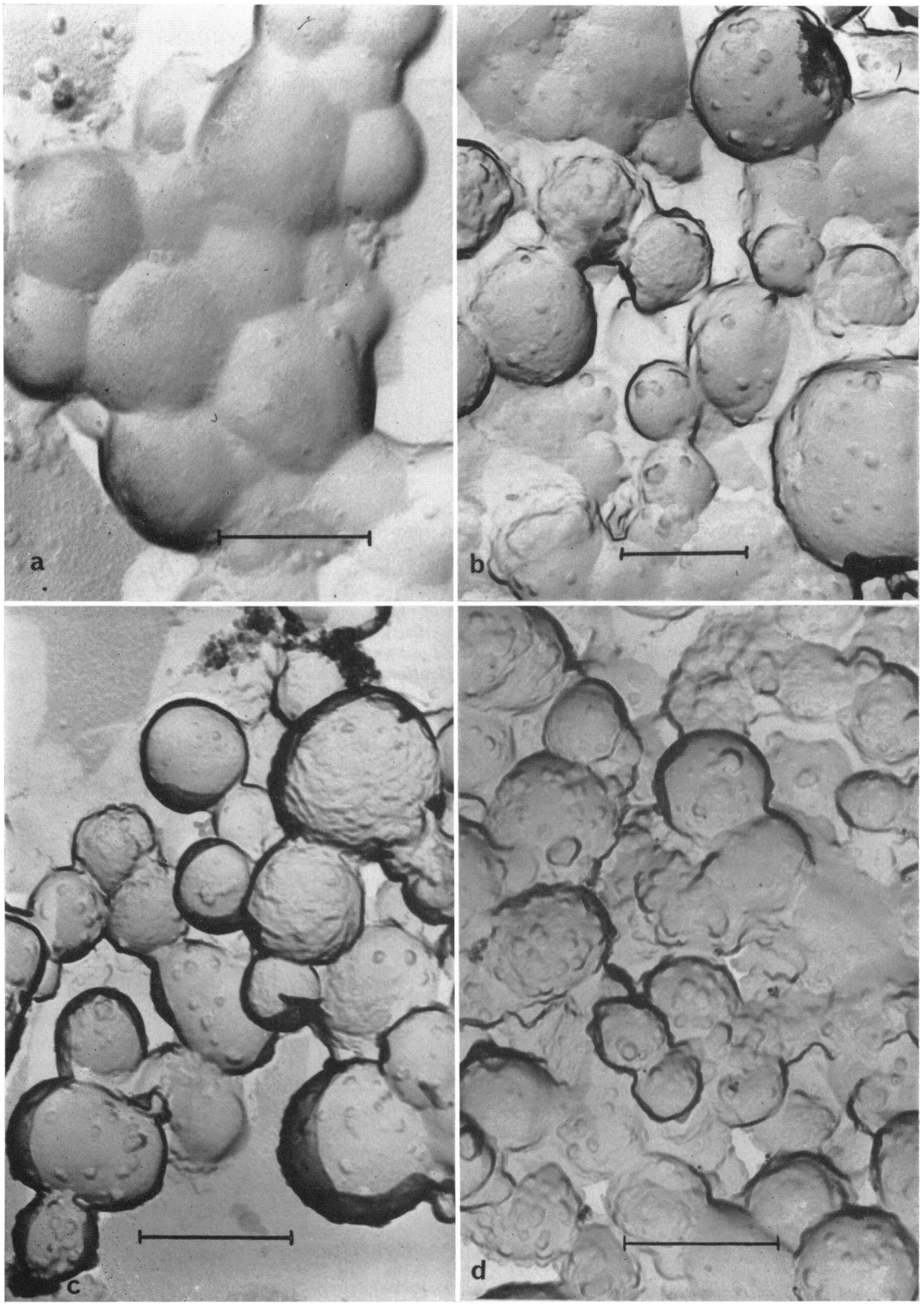

FIG. 4. *Inactivation of native PHB granules by trypsin (a, c, and d, 25,000×; b, 20,000×). Preparations were treated with trypsin for (a) 0, (b) 15, (c) 30, and (d) 60 min. The reaction mixtures contained native PHB granules (4.0 mg), Tris-HCl buffer, pH 8.0 (15 μmoles), $CaCl_2$ (2 μmoles), and trypsin (50 μg) in a final volume of 0.6 ml; incubation temperature was 33 C. After incubation for 0, 15, 30, and 60 min, respectively, ovomucoid (200 μg) was added, and the reaction mixtures were cooled to 0 C. The per cent inactivation was (a) 0, (b) 12, (c) 70, and (d) 83. In untreated preparations, 90% of the PHB is hydrolyzable by the depolymerizing enzymes.*

mainly characterized by membrane fragmentation, loss of coalescence, and surface alterations. These observations suggest that native PHB granules possess definite structural features, and that disruption of these structures results in decreased susceptibility of the polymer to enzymatic hydrolysis.

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