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Effect of Polymyxin B, Tyrocidine, Gramicidin D, and Other Antibiotics on the Enzymatic Hydrolysis of Poly-β-Hydroxybutyrate

J. M. MERRICK[1]

Department of Biochemistry, School of Medicine, State University of New York at Buffalo, Buffalo, New York

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ABSTRACT

MERRICK, J. M. (State University of New York, Buffalo). Effect of polymyxin B, tyrocydine, gramicidin D, and other antibiotics on the enzymatic hydrolysis of poly-β-hydroxybutyrate. J. Bacteriol. **90**:965-969. 1965.—Previous studies have demonstrated that native poly-β-hydroxybutyrate (PHB) granules isolated from *Bacillus megaterium* are surrounded by a discrete membranelike structure. Morphological alterations of PHB granules are mainly characterized by membrane fragmentation, and can be correlated with decreased susceptibility of the polymer to enzymatic hydrolysis by soluble factors. In the present investigation, the inhibitory effect of a variety of surface-active and other antibiotics on the enzymatic depolymerization of PHB was examined. The most potent inhibitors were polymyxin B, tyrocidine, and gramicidin D. These polypeptide antibiotics are known to attack other types of membranous structures. The results, therefore, support previous evidence that the membrane or similar constituents of PHB granules are intimately involved in its metabolism. Chlortetracycline was also found to be a potent inhibitor of the depolymerization, but its mechanism of action may be different from the other antibiotics. The polymer-synthesizing enzyme(s), also localized on the granules, is inhibited by tyrocidine and gramicidin D but not by polymyxin B or chlortetracycline.

Poly-β-hydroxybutyrate (PHB), the lipid storage material of many bacteria, occurs in the form of intracellular granules. Electron microscopy of isolated granules (Lundgren, Pfister, and Merrick, 1964), as well as of thin sections of *Bacillus cereus* and *Rhodospirillum rubrum* (Pfister and Lundgren, 1964; Boatman, 1964), has demonstrated that such granules are bounded by a discrete membrane-like structure. Further studies have also indicated that isolated native PHB granules possess definite structural features and that disruption of morphological integrity can be correlated with decreased susceptibility of PHB granules to enzymatic hydrolysis (Lundgren et al., 1964; Merrick, Lundgren, and Pfister, 1965). Thus, granules inactivated by various agents (organic solvents, proteolytic enzymes, centrifugation, and freezing and thawing) show morphological changes that are mainly characterized by membrane fragmentation, loss of coalescence, and surface alterations. Although it appears highly probable that the membrane is intimately involved in the metabolism of PHB, its specific role in the breakdown (or synthesis) of the polymer is not known. In addition to the labile particulate component of the granules, two soluble factors are required for the depolymerization of PHB. These consist of a heat-stable "activator" and a heat-labile "depolymerase" whose successive action on the granules results in the breakdown of the polymer to β-hydroxybutyric acid and small amounts of soluble esters. Both of these factors appear to be proteins (Merrick and Doudoroff, 1964). Under certain conditions, trypsin can replace the activator (Merrick and Doudoroff, 1964).

Certain antibiotics (polymyxin B, tyrocidine) have been known to exert their toxicity on bacteria by interaction with the cytoplasmic membrane, causing its disorganization with concomitant leakage of the intracellular content (Davis and Feingold, 1962; Newton, 1956; Hotchkiss, 1944). These antibiotics, as well as others (oligomycin, gramicidin D), have also been shown to interact with intracellular membranous structures (mitochondria) and to do so in a highly

[1] Present address: Department of Bacteriology and Botany, Syracuse University, Syracuse, N.Y.

specific manner (Neubert and Lehninger, 1962; Weinstein, Scott, and Hunter, 1964). Since the membrane of PHB and metabolism of the polymer may be closely related, a variety of surface-active antibiotics and others were examined for their effect on the depolymerization of PHB.

MATERIALS AND METHODS

The general methods for isolation of native PHB granules from *B. megaterium* (strain Km) and for the preparation of the soluble depolymerizing enzyme system (activator and depolymerase) from *R. rubrum* (strain III, C. B. van Niel) have been previously described (Merrick and Doudoroff, 1964).

The standard reaction mixtures for measurement of depolymerization (turbidity disappearance) contained 1.0 mg of PHB granules, 5.0 μmoles of $CaCl_2$, and 125 μmoles of tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (*p*H 8.0) in a total volume of 5.0 ml. Activation of PHB granules was achieved by preincubating the mixture with activator (52 μg) for 30 min and was followed by the addition of depolymerase (3.9 μg). Incubation temperature was 33 C, and the reaction was followed by measuring the decrease in turbidity in a Klett colorimeter (red filter, no. 66). Antibiotics dissolved in water or alcohol were added at the beginning of the activation of the PHB granules. In the case of alcohol-soluble antibiotics, control experiments with alcohol, but in the absence of antibiotic, were also carried out. The hydrolysis of PHB and its inhibition were determined 30 min after the addition of depolymerase.

Synthesis of PHB from D(−)-β-hydroxybutyryl-coenzyme A was determined as previously described (Merrick and Doudoroff, 1961). PHB granules and antibiotic were incubated for 5 min at 33 C before initiating the reaction by addition of D(−)-β-hydroxybutyryl-coenzyme A.

In a preliminary screening, antibiotics which did not give inhibition in concentrations of 1.0 mg per 5.0 ml of the incubation mixture were not further examined.

The preparations of antibiotics used were chlortetracycline, tetracycline, and puromycin (Lederle Laboratories, Pearl River, N.Y.); novobiocin (The Upjohn Co., Kalamazoo, Mich.); erythromycin and vancomycin (Lilly Research Laboratories, Indianapolis, Ind.,); penicillin G, nystatin, and streptomycin (Squibb Institute for Medical Research, New Brunswich, N.J.); oxytetracycline (Chas. Pfizer & Co., Inc., New York, N.Y.); gramicidin D, bacitracin, colistimethate sodium, colistin sulfate, fungimycin and neomycin (Warner-Lambert Research Institute, Morris Plains, N.J.); oligomycin and antimycin A (Wisconsin Alumni Research Foundation); polymyxin B (Burrows Wellcome & Co., New York, N.Y.), spiramycin (Ives Cameron); chloramphenicol (Parke, Davis & Co., Detroit, Mich.) and kanamycin (Bristol Laboratories, Inc., Syracuse, N.Y.). Tyrocidine hydrochloride and D-cycloserine were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

TABLE 1. *Inhibition of depolymerization of PHB by antibiotics**

Antibiotic	Concn giving 50% inhibition
	M
Polymyxin B	1.75×10^{-6}
Tyrocidine	2.25×10^{-6}
Gramicidin D	3.75×10^{-6}
Chlortetracycline	5.38×10^{-6}
Colistin	1.7×10^{-5}
Tetracycline	$>10^{-5}$
Oxytetracycline	$>10^{-4}$
Antimycin A	$>10^{-4}$
Oligomycin	$>10^{-4}$
Streptomycin	$>10^{-4}$
Fungimycin	$>10^{-4}$

* The conditions of incubation and assay are described in the text. The following antibiotics were not inhibitory: bacitracin, puromycin, chloramphenicol, neomycin, kanamycin, spiramycin, penicillin, D-cycloserine, novobiocin, erythromycin, vancomycin, nystatin, colistimethate.

RESULTS

A large number of antibiotics were tested for their ability to inhibit the enzymatic depolymerization of PHB. The results of these studies are summarized in Table 1. The data are expressed in terms of the molar concentration of antibiotic required to give 50% inhibition and were obtained by testing antibiotics at different concentrations and interpolating the concentration required for 50% inhibition. It can be seen that of the antibiotics tested the polypeptide antibiotics, polymyxin B, tyrocidine, and gramicidin D, are potent inhibitors of depolymerization. Chlortetracycline, the only nonpolypeptide antibiotic of this series is also a potent inhibitor. Ca^{2+} is required for the observed inhibitory effects of chlortetracycline and is not replaceable by Mg^{2+}. This dependence was not noted in the case of the polypeptide antibiotics.

The details of typical assays of the inhibitory effect of polymyxin B, tyrocidine, gramicidin D, and chlortetracycline are given in Fig. 1, which shows inhibition as a function of time with varying concentration of the antibiotic. The effect of antibiotic concentration on inhibition of depolymerization of PHB is shown in Fig. 2. The results obtained (except, perhaps, with gramicidin D) suggest that the antibiotics may be combining stoichiometrically with some sensitive structure of the granules. The inhibition curve observed

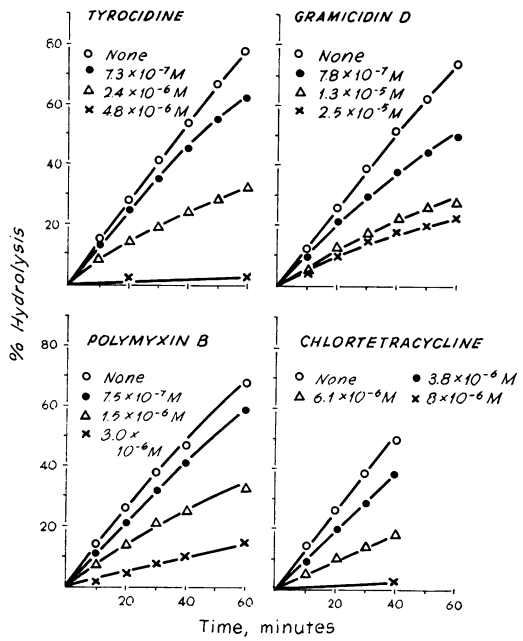

FIG. 1. *Inhibitory effect of polymyxin B, tyrocidine, gramicidin D, and chlortetracycline on the enzymatic depolymerization of PHB. The conditions of incubation and assay are described in the text.*

with increasing concentrations of gramicidin D is possibly a reflection of its limited solubility, and the sigmoid shape of the curve observed with chlortetracycline may be due to preferential adsorption of the antibiotic by components other than sensitive structures.

Replacement of activator by trypsin in the depolymerizing system gave essentially similar results.

Interaction of antibiotic with PHB granules was demonstrated by binding experiments (Table 2). Binding of antibiotic by PHB granules was determined by adding PHB granules to a solution of the antibiotic, followed by removing PHB by centrifugation and measuring the inhibitory capacity of the supernatant fluid. In each case, PHB granules effectively adsorbed the antibiotic, as indicated by markedly reduced inhibitory capacity of the supernatant fluid. The treated granules were washed several times by suspension in buffer, followed by centrifugation. Since PHB granules are inactivated by repeated centrifugation, special procedures previously described by Merrick and Doudoroff (1964) were utilized. After washing, polymyxin B- and tyrocidine-treated granules were inactive to the depolymerizing enzyme system, whereas chlortetracycline-treated PHB granules were effectively hydrolyzed. Thus, chlortetracycline is not as firmly bound to the granules as the polypeptide antibiotics.

Since the PHB synthetic enzyme system has previously been shown to be associated with native PHB granules (Merrick and Doudoroff, 1961), it was of interest to see whether the inhibitory antibiotics also disrupted synthetic capacity of PHB granules. The results of such experiments are shown in Table 3. Both tyrocidine and gramicidin D are effective inhibitors of synthesis, whereas polymyxin B and chlortetracycline (in the presence of $MgCl_2$ or $CaCl_2$) were without effect under the experimental conditions. These data therefore support previous evidence that the enzyme associated with synthesis is not identical to the labile particulate component of the depolymerizing system (Merrick and Doudoroff, 1964), and also indicate some differences in the mechanism of interaction of PHB granules with the various antibiotics.

DISCUSSION

The results of experiments reported in this paper show that, of a number of antibiotics tested, several are effective inhibitors of the enzymatic depolymerization of PHB. The most potent inhibitors (polymyxin B and tyrocidine) are polypeptides which have previously been shown to act on cell membranes, causing disorganization and resultant leakage of cellular constituents (Davis and Feingold, 1962; Newton, 1956; Hotchkiss, 1944). It has not been clearly established whether the inhibitory action of gramicidin D on bacteria is due to its surface-active properties but this antibiotic, as well as tyrocidine, is known to attack other types of membranes (Neubert and Lehninger, 1962; Weinstein et al.,

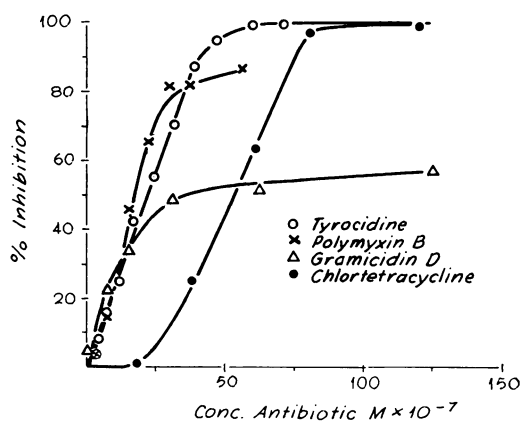

FIG. 2. *Effect of concentration of antibiotics on depolymerization of PHB. The conditions of incubation and assay are described in the text.*

TABLE 2. *Binding of antibiotics by PHB granules**

Expt	Antibiotic	Inhibition	Concn of antibiotic in assay system ($\times 10^{-6}$)	Antibiotic adsorbed
		%	M	%
1	Tyrocidine alone	100	4.64	
	Tyrocidine + PHB granules (supernatant fluid)	16	0.75†	83.8
2	Polymyxin B alone	56	1.94	
	Polymyxin B + PHB granules (supernatant fluid)	8	0.5†	75.6
3	Chlortetracycline alone	59	5.98	
	Chlortetracycline + PHB granules (supernatant fluid)	10	2.75†	54.0

* The complete incubation mixtures contained the following components: experiment 1, PHB (18 mg) and Tris-HCl buffer, *p*H 8.0 (14 μmoles); experiments 2 and 3, PHB (18 mg), $CaCl_2$ (4.0 μmoles), and Tris-HCl buffer, *p*H 8.0 (18 μmoles). Concentrations of antibiotics in experiments 1, 2, and 3 were 1.65×10^{-4} M (tyrocidine), 1.08×10^{-4} M (polymyxin B), and 2.14×10^{-4} M (chlortetracycline), respectively. Final volume was 1.0 ml. Incubation was conducted at 0 C for 10 min in experiment 1, and for 20 min in experiments 2 and 3. Controls were identical to the above but without PHB granules. The granules were removed by centrifugation and samples of the treated and untreated antibiotic solutions were examined for their capacity to inhibit the enzymatic depolymerization of PHB under the assay conditions described in the text.

† Calculated from Fig. 2.

TABLE 3. *Effect of antibiotics on synthesis of PHB**

Antibiotic	C^{14} Incorporated into PHB (% of control)
Tyrocidine, 2.4×10^{-6} M	3
Gramicidin D, 5.6×10^{-6} M	19
Polymyxin B, 1.8×10^{-6} M	100
Chlortetracycline, 1.5×10^{-5} M	100

* The reaction mixtures contained PHB granules (57.5 μg of PHB and 2.6 μg of protein per ml), 0.1 μeq of D(−)-β-hydroxybutyryl (C^{14})-coenzyme A per ml, plus antibiotic in the concentrations indicated, and the following components: magnesium chloride, 12.5 μmoles/ml; and potassium phosphate buffer, *p*H 7.2, 125 μmoles/ml. Incubation was for 10 min at 33 C. The reactions were stopped by addition of alcohol (70% final concentration).

1964). Thus, Neubert and Lehninger (1962) have demonstrated that mitochondrial swelling is greatly stimulated by these antibiotics and they have suggested that this effect may be due to a specific interaction of the polypeptide with a sensitive structure of the mitochondrion. Presumably, the polypeptide antibiotics also interfere with the depolymerization of PHB by interacting with the PHB membrane and causing its disorganization. It is noteworthy that some of the other antibiotics which inhibit depolymerization, albeit at much higher concentrations, have also been implicated in altered metabolism of other membranous structures, e.g., oligomycin (Neubert and Lehninger, 1962), streptomycin (Davis and Feingold, 1962), and colistin (Kaye and Chapman, 1963).

Of particular interest is the inhibition of depolymerization by the tetracyclines, chlortetracycline being the most effective of the three studied. The mechanism of action of this drug is not clear, although its chelating properties have been implicated (Weinberg, 1954). In the present case, inhibition of depolymerization is not observed unless Ca^{2+} is present. As has been previously postulated (Goldman, 1960) the metal chelate of chlortetracycline may be the effective inhibitor.

Our previous results (Merrick and Doudoroff, 1964) demonstrated that PHB granules can be treated with various physical and chemical agents which result in preparations no longer suitable as a substrate for the soluble depolymerizing enzyme system. These granules show structural alterations mainly characterized by membrane fragmentation (Merrick et al., 1965). It is clear from the results reported here that substances known to interfere with membrane structural organization also inhibit the depolymerization process. This correlation supports previous evidence that the membrane or similar constituents of the PHB granule are intimately involved in its metabolism.

ACKNOWLEDGMENT

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LITERATURE CITED

BOATMAN, E. S. 1964. Observations on the fine structure of spheroplasts of *Rhodospirillum rubrum*. J. Cell. Biol. **20**:297–311.

DAVIS, B. D., AND D. S. FEINGOLD. 1962. Antimicrobial agents: mechanism of action and use in metabolic studies, p. 343–397. *In* I. C. Gunsalus and R. Y. Stanier [ed.], The bacteria, vol. 4. Academic Press, Inc., New York.

GOLDMAN, D. S. 1960. The inhibition of alanine dehydrogenase by metal chelates of tetracycline. J. Biol. Chem. **235**:616–619.

HOTCHKISS, R. D. 1944. Gramicidin, tyrocidine, and tyrothyricin. Advan. Enzymol. **4**:153–199.

KAYE, J. J., AND G. B. CHAPMAN. 1963. Cytological aspects of antimicrobial antibiosis. III. Cytologically distinguishable stages in antibiotic action of colistin sulfate on *Escherichia coli*. J. Bacteriol. **86**:536–543.

LUNDGREN, D. G., R. M. PFISTER, AND J. M. MERRICK. 1964. Structure of poly-β-hydroxybutyric acid granules. J. Gen. Microbiol. **34**: 441–446.

MERRICK, J. M., AND M. DOUDOROFF. 1961. Enzymatic synthesis of poly-β-hydroxybutric acid in bacteria. Nature **189**:890–892.

MERRICK, J. M., AND M. DOUDOROFF. 1964. Depolymerization of poly-β-hydroxybutyrate by an intracellular enzyme system. J. Bacteriol. **88**:60–71.

MERRICK, J. M., D. G. LUNDGREN, AND R. M. PFISTER. 1965. Morphological changes in poly-β-hydroxybutyrate granules associated with decreased susceptibility to enzymatic hydrolysis. J. Bacteriol. **89**:234–239.

NEUBERT, D., AND A. L. LEHNINGER. 1962. The effect of oligomycin, gramicidin and other antibiotics on reversal of mitochondrial swelling by adenosine triphosphate. Biochim. Biophys. Acta **62**:556–565.

NEWTON, B. A. 1956. Properties and mode of action of the polymyxins. Bacteriol. Rev. **20**:14–27.

PFISTER, R. M., AND D. G. LUNDGREN. 1964. Electron microscopy of polyribosomes within *Bacillus cereus*. J. Bacteriol. **88**:1119–1129.

WEINBERG, E. D. 1954. The reversal of the toxicity of oxytetracycline by multivalent cations. J. Infect. Diseases **95**:291–301.

WEINSTEIN, J., A. SCOTT, AND F. E. HUNTER, JR. 1964. The action of gramicidin D on isolated liver mitochondria. J. Biol. Chem. **239**:3031–3037.