66, 375.
Gigliotti, H. J., and Levenberg, B. (1964), *J. Biol. Chem. 239*, 2274.
Goldenberg, J. M., Faber, M., Alston, E. M., and Chargaff, E. C. (1949), *Science 109*, 534.
Hinman, R. L., and Fulton, D. (1958), *J. Am. Chem. Soc. 80*, 1895.
Kolthoff, I. M. (1924), *J. Am. Chem. Soc. 46*, 2009.
Lichtenstein, J., and Gertner, S. (1942), *J. Am. Chem. Soc. 64*, 1021.
Lineweaver, J., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
McKennis, H., Jr., and Yard, A. S. (1954), *Anal. Chem. 26*, 1960.
McKennis, H., Jr., Yard, A. S., Weatherby, J. H., and Hagy, J. A. (1959), *J. Pharmacol. Exptl. Therap. 126*, 109.
Meister, A. (1962), *Enzymes 6*, 443.
O'Brien, R. D., Kirkpatrick, M., and Miller, P. S. (1964), *Toxicol. Appl. Pharmacol. 6*, 371.
Pamiljans, V., Krishnaswamy, P. R., Dumville, G., and Meister, A. (1962), *Biochemistry 1*, 153.
Speck, J. F. (1949), *J. Biol. Chem. 179*, 1405.
Wilkinson, G. N. (1961), *Biochem. J. 80*, 324.

Purification and Properties of a D(−)-β-Hydroxybutyric Dimer Hydrolase from *Rhodospirillum rubrum**

J. M. Merrick and Chi Ing Yu

ABSTRACT: An enzyme which catalyzes the hydrolysis of the dimeric ester of D(−)-β-hydroxybutyric acid to 2 equiv of the monomer has been purified 70-fold from *Rhodospirillum rubrum*. It exhibits a high degree of specificity for β-hydroxybutyric dimer possessing the D(−) configuration. Evidence is presented which suggests that the dimer may not be the physiological substrate for the enzyme. Thus, the hydrolase attacks a low molecular weight oligomer of β-hydroxybutyric acid, presumably the trimer, at much faster rates than the dimer. The enzyme shows no activity with other esters tested.

Poly-β-hydroxybutyrate (PHB),[1] the major lipid reserve material of many types of bacteria, occurs in the cell as discrete granules. Recent investigations have demonstrated that the enzymatic depolymerization of native PHB granules isolated from *Bacillus megaterium* is dependent on the successive action of two soluble factors (tentatively designated as activator and depolymerase) isolated from extracts of *Rhodospirillum rubrum* (Merrick and Doudoroff, 1964). In addition to the soluble enzyme system, a labile particulate factor intimately bound to the granules also contributes to the depolymerization of PHB. Evidence has been presented which suggests that this labile factor may be associated with the membrane of the PHB granules (Merrick *et al.*, 1965; Merrick, 1965). The products of depolymerization are D(−)-β-hydroxybutyrate (80–85%) and a soluble ester (15–20%). The soluble ester has been isolated and its identification as the dimeric ester of D(−)-β-hydroxybutyric acid (3-*O*-D-(3-D-hydroxybutyryl)hydroxybutyric acid) is reported in this paper.

An extracellular enzyme isolated from *Pseudomonas lemoignei* is also capable of digesting PHB, but the mechanism of hydrolysis differs from the enzyme system described above (Merrick *et al.*, 1962; Merrick and Doudoroff, 1964; Delafield *et al.*, 1965b). The exoenzyme attacks purified PHB but not native PHB granules, while the activator and depolymerase fractions from *R. rubrum* do not digest the purified polymer. Further, the action of the extracellular depolymerase on the polymer results in the formation of the dimeric ester of β-hydroxybutyric acid as the principal product, although some monomer is also produced.

Thus, the dimer appears to be an important metabolite in the breakdown of purified PHB or PHB contained in native granules. The further metabolism of the dimer to its monomeric constituents can readily be demonstrated with *R. rubrum* extracts. The present communication describes the purification and proper-

* From the Biological Research Laboratories, Department of Bacteriology and Botany, Syracuse University, Syracuse, New York, and Department of Biochemistry, School of Medicine, State University of New York at Buffalo, New York. *Received February 10, 1966; revised August 29, 1966.* This investigation was supported by Research Grants AI-04501 and AI-07176 from the National Institutes of Health, U. S. Public Health Service.

[1] Abbreviations: PHB, poly-β-hydroxybutyrate; NAD, nicotinamide-adenine dinucleotide; DFP, diisopropylphosphorofluoridate.

ties of the dimer hydrolytic enzyme[2] from *R. rubrum*. The properties of the dimer hydrolase from *P. lemoignei* have recently been reported (Delafield *et al.*, 1965a).

Experimental Procedure

Materials and Methods. Cultures of *R. rubrum* (strain 111, C. B. van Niel) were grown on acetate medium in the light (Shuster and Doudoroff, 1962). Polymer-depleted cells were obtained by allowing cultures to incubate in the light in the presence of CO_2 for 18–20 hr after the exhaustion of acetate. The cells were washed with 0.05 M Tris–HCl (pH 8.0) and stored as a cell paste at −20°.

The dimeric ester of D(−)-β-hydroxybutyric acid was prepared by the enzymatic digestion of purified poly-β-hydroxybutyrate with the extracellular depolymerase from *P. lemoignei* (Merrick *et al.*, 1962; Delafield *et al.*, 1965b) or by chemical synthesis by procedures previously described (Olsen *et al.*, 1965). The latter procedures were also utilized for the synthesis of the other possible stereoisomers of the dimer. Native PHB granules were prepared as described by Merrick and Doudoroff (1964). Purified PHB was obtained by extraction of native PHB granules with chloroform followed by precipitation of the polymer with alcohol. The purified polymer was washed successively with alcohol, ether, and ligroin, and finally dried under vacuum.

The soluble ester obtained from the digestion of native PHB granules by the purified *R. rubrum* enzyme systems was isolated and identified in the following manner. Native PHB granules isolated from *B. megaterium* were depolymerized with purified activator and depolymerase as previously described (Merrick and Doudoroff, 1964). The reaction was carried out in a Radiometer pH-Stat and was maintained at pH 8.0. When no further alkali was consumed the products of the reaction were converted to the free acids with Dowex 50(H^+) (200–400 mesh) followed by continuous extraction of the acids in ether for 8 hr. The ether extract was evaporated and the remaining syrup was dissolved in chloroform. Paper chromatography was carried out in the following solvent systems: (A) ether–benzene–formic acid (50:50:12.5, v/v); (B) 1-butanol saturated with 1.5 N aqueous ammonia; and (C) 1-butanol–ethylamine (Manganelli and Brofazi, 1957). Only two acid spots corresponding to β-hydroxybutyrate and the dimeric ester of β-hydroxybutyric acid were found. Separation of the two acids was achieved by silicic acid column chromatography (Olsen *et al.*, 1965). The purified dimer was shown to be homogenous by paper chromatography in solvent systems A–D (1-butanol–water–ethanol (50:40:19, v/v)). An R_F value identical with chemically synthesized dimer was obtained in all the solvent systems. The yield of dimer was 10% of the initial polymer; $[\alpha]_D^{25}$ −20.0° (*c* 0.32, H_2O).

Anal.[3] Calcd for $C_8H_{14}O_5$ (mol wt 190): C, 50.53; H, 7.37. Found: C, 50.30; H, 7.42.

A small sample of a low molecular weight oligomer of β-hydroxybutyric acid, tentatively identified as the trimeric ester of D-β-hydroxybutyric acid, was kindly provided by M. Doudoroff. All other chemicals were commercial preparations.

Total β-hydroxybutyric acid (free and esterified acid) was determined by the method of Slepecky and Law (1960). β-hydroxybutyric acid was determined with a specific D(−)-hydroxybutyrate dehydrogenase, purified as described by Delafield *et al.* (1965a). The reaction was allowed to proceed to equilibrium and the monomer concentration was calculated using the partial equilibrium constant $K_{eq} = 0.13$ under the conditions of the assay (Shuster and Doudoroff, 1962). Dimer concentration was determined by the alkaline hydroxylamine reaction of Hestrin (1949).

Enzyme Assay. The following mixtures were used for measurement of dimer hydrolase activity (in micromoles): Tris–HCl buffer, pH 8.0 (25); dimer (1.66); and enzyme. Final volume was 0.2 ml. The mixture was incubated for 30 min at 30° and the reaction stopped by heating for 3 min in a boiling water bath. The amount of monomer formed was measured with D(−)-β-hydroxybutyrate dehydrogenase as described above. Controls included tubes without enzyme and with heat-inactivated enzyme. A unit of hydrolase activity is defined as that amount of enzyme which catalyzes the formation of 1 μmole of β-hydroxybutyrate in 30 min under the above conditions. Occasionally, the reactions were followed by titration of the acid released with a Radiometer pH-Stat. Protein was determined by the method of Lowry *et al.* (1951).

Purification and Properties of the Dimer Hydrolase. Extracts of *R. rubrum* (starting material was 14 g of cell paste, wet weight) were prepared by sonic oscillation of a 15% (w/v) suspension of packed cells in 0.05 M Tris–HCl buffer, pH 8.0, The cell suspension in 35–50-ml portions was disrupted in a 20-kcycle Bronwill Biosonik sonic oscillator for 10 min. The extract was centrifuged for 10 min at 27,000*g* and the supernatant fluid centrifuged again in a Spinco Model L ultracentrifuge for 120 min at 60,000*g*. All subsequent operations were performed at 0° and centrifugations were carried out at 27,000*g* for 10 min. The supernatant fluid was partially freed of nucleic acids by precipitation with one-tenth volume of 0.5 M $MnCl_2$. After a period of not less than 3 hr, the mixture was centrifuged and the precipitate was discarded. Solid ammonium sulfate was added to the supernatant fluid to bring the final

[2] Since most of the studies reported in this paper were carried out with D(−)-β-hydroxybutyric acid dimer, the enzyme was conveniently referred to as a dimer hydrolase. However, specificity studies described in the text suggest that low molecular weight oligomers of β-hydroxybutyric acid may, in fact, be better substrates for the enzyme than the dimer. Until such substances become available in substrate quantities for further studies, it seems appropriate nevertheless to delay a more correct designation of the enzyme.

[3] The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

TABLE I: Purification of Dimer Hydrolase.

Fraction	Total Act. (units)	Total Protein (mg)	Sp Act. (units/mg)	Yield (%)
(1) Crude extract, 27,000*g* supernatant fluid	665	3850	0.17	100
(2) Supernatant fluid, 2 hr, 60,000*g*	583	1620	0.36	88
(3) $MnCl_2$ ppt, $(NH_4)_2SO_4$ ppt, 0–50% satn	600	485	1.2	90
(4) DEAE-cellulose column eluate, $(NH_4)_2SO_4$ ppt, 0–90% satn	347	66.5	5.2	52
(5) Hydroxylapatite column eluate	114	9.7	11.8	17

concentration to 50% saturation. After 20 min, the turbid suspensions was centrifuged and the precipitate was dissolved in 0.05 of the inital volume of 0.01 M potassium phosphate buffer at pH 7.0. The solution was dialyzed overnight against the same buffer. After dialysis the ammonium sulfate fraction (13.2 ml) was applied directly to a DEAE-cellulose column (diameter, 2.0 cm; bed volume, 97 ml), which had previously been equilibrated with 0.01 M potassium phosphate buffer, pH 7.0. The column was washed with 0.01 M phosphate buffer, pH 7.0 (150 ml), and the tubes containing depolymerase activity (Merrick and Doudoroff, 1964) were pooled, lyophilized, and stored at −20°. The DEAE-cellulose column at this step must be exhaustively washed with the 0.01 M phosphate buffer to remove traces of depolymerase. Unless this special precaution was taken, purified hydrolase obtained in the subsequent step was contaminated with a small amount of depolymerase. Such preparations of hydrolase exhibited hydrolytic activity toward native PHB granules. After all the depolymerase activity had been removed, the column was washed with 0.1 M Tris–HCl buffer, pH 8.0 (150 ml). Hydrolase activity was then eluted with 0.1 M NaCl in 0.1 M Tris–HCl buffer, pH 8.0. Fractions (5 ml) were collected and the activity appeared after approximately 75 ml of eluate had passed through the column. The next 80 ml was collected and contained 60% of the enzyme put on the column, with a four- to fivefold increase in specific activity. An additional fraction containing hydrolase activity could be eluted by increasing the salt concentration in 0.1 M Tris–HCl buffer, pH 8.0, to 0.4 M. This fraction contained less than 10% of the enzymatic activity put on the column, with no increase in specific activity, and was not further studied. The pooled eluate was made 90% saturated with respect to ammonium sulfate and the precipitate that formed in 30 min was collected by centrifugation. After solution in 0.01 M phosphate buffer, pH 7.0, and dialysis against the same buffer overnight (final volume, 19.0 ml), the protein was applied to the top of a hydroxylapatite column (diameter, 1 cm; bed volume, 8.0 ml), which had previously been equilibrated with 0.01 M potassium phosphate buffer, pH 7.0. The column was washed with 0.01 M phosphate buffer, pH 7.0 (42.0 ml), and hydrolase activity was eluted with 0.02 M phosphate buffer, pH 7.0. Fractions (3 ml) were collected and the enzyme appearing in fractions 20–40 were pooled, lyophilized, and stored at −20°. The over-all purification was usually approximately 70–75-fold with 20% recovery of total enzyme units. Increasing the molarity of the buffer to 0.04 and 0.08 M resulted in the elution of additional fractions containing hydrolase activity. These fractions contained less than 10% of the enzymatic activity put on the column with no increase in specific activity and were not further studied. A summary of a typical purification is presented in Table I.

The effect of incubation time, protein concentration, and pH on the reaction velocity are illustrated in Figures 1 and 2. The K_m calculated according to Lineweaver and Burk (1934) was 2.48×10^{-4} M. The purified enzyme was stable for several months at −20°.

TABLE II: Stoichiometry of Dimer Hydrolase Reactions.[a]

Compound	Concn (μmoles/incubn mixture)
Dimer	−0.68
β-Hydroxybutyrate	+1.33

[a] The reaction mixture contained (in micromoles): dimer, 1.66; Tris–HCl buffer, pH 8.0, 25; and 120 μg of protein. Final volume, 0.2 ml; incubation time was 30 min at 30°.

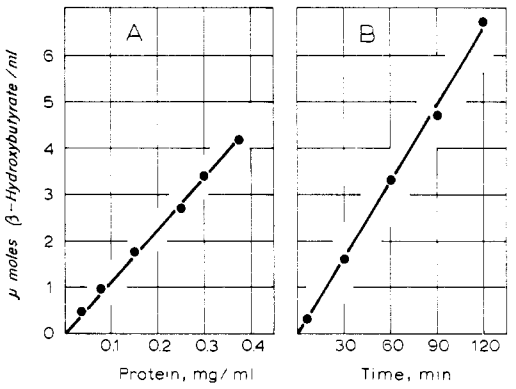

FIGURE 1: Effect of enzyme concentration (A) and incubation time (B) on β-hydroxybutyrate formation. Conditions as in standard assay. In B, reaction mixtures contained 28 μg of protein.

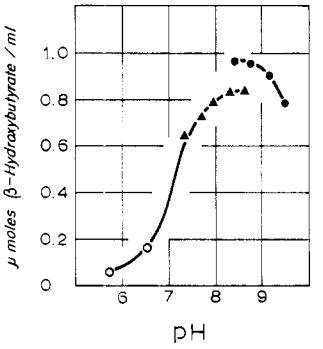

FIGURE 2: Effect of pH on the reaction velocity. Conditions as in the standard assay but with the following buffers: ○—○, Tris–maleic; ▲—▲, Tris–HCl; and ●—●, 2-amino-2-methyl-1,3-propanediol. The dimer was adjusted to pH 7.0 before use and the pH values are those observed after incubation. Reaction mixtures contained 15 μg of protein.

Stoichiometry. As shown in Table II, the hydrolase catalyzes the stoichiometric conversion of the dimer to 2 equiv of β-hydroxybutyrate. At the end of 2 hr the dimer was completely hydrolyzed to the monomer (3.32 μmoles).

For the further identification of the products of hydrolysis, reaction mixtures identical with the one described above were prepared and incubated for various periods of time. Following acidification, the acids were extracted into ether, concentrated, and examined by paper chromatography in solvent systems A–C. Only two acidic spots were observed, one corresponding to that of β-hydroxybutyric acid and the other corresponding to the dimeric ester. When the incubation period was long enough for complete hydrolysis, only one spot corresponding to the monomer was observed.

Specificity Studies. Specificity studies carried out with the dimer hydrolase are shown in Table III. The purified hydrolase was examined for its reactivity with the dimeric ester of β-hydroxybutyrate (3-*O*-D-(3-D-hydroxybutyryl)hydroxybutyric acid) prepared by three different methods: (a) enzymatic degradation of purified PHB by the *P. lemoignei* extracellular enzyme, (b) enzymatic digestion of native PHB granules by purified extracts of *R. rubrum*, and (c) chemical synthesis. Similar results with the differently prepared dimers were obtained, providing further evidence for their identical nature. The enzyme exhibits a high degree of stereospecificity for the D(−)-β-hydroxybutyric acid dimeric ester. It does however attack 3-*O*-L-(3-D-hydroxybutyryl)hydroxybutyric acid, but at a considerably reduced rate. Of special interest was the reactivity of the hydrolase with the presumed trimeric ester of D-β-hydroxybutyric acid. Much higher rates of hydrolysis were obtained with this substrate than with the dimer. This result suggests that the dimer may not be the physiological substrate for the hydrolase and that low molecular weight oligomers of β-hydroxybutyrate may play an important role in the depolymerization of the polymer. However we have not as yet detected any soluble esters other than the dimer as intermediates in the breakdown of

TABLE III: Specificity of Dimer Hydrolase.[a]

Substrate	μmoles of Acid Produced/ 30 min
Dimer (DD) (*P. lemoignei*)[b]	0.43
Dimer (DD) (*R. rubrum*)[b]	0.43
Dimer (DD) (chemical)[b]	0.43
Dimer (DL) (chemical)[c]	0.0
Dimer (LD) (chemical)[d]	0.07
Dimer (LL) (chemical)[e]	0.0
Trimer (DDD) (*P. lemoignei*)[b]	1.84
Ethyl DL-β-hydroxybutyrate	0.0
Native PHB granules[b]	0.0
Purified PHB[b]	0.0

[a] Reactions were carried out in a Radiometer pH-Stat and a constant pH of 8.0 was maintained by the addition of potassium hydroxide. Concentration of dimer, trimer, or PHB was based on total β-hydroxybutyrate content and corresponded to 16 μmoles of the monomer. Unless indicated otherwise reactions were initiated by the addition of hydrolase (62 μg). Initial volume of the reaction mixtures was 1.0 ml; temperature was 30°. The following esters were not hydrolyzed by the hydrolase: tributyrin, triacetin, methyl acetate, methyl butyrate, acetylcholine, and *p*-tosylarginine methyl ester. [b] See text for explanation of various preparations. [c] DL = 3-*O*-D-(3-L-hydroxybutyryl)hydroxybutyric acid. [d] LD = 3-*O*-L-(3-D-hydroxybutyryl)hydroxybutyric acid. [e] LL = 3-*O*-L-(3-L-hydroxybutyryl)hydroxybutyric acid.

native PHB granules isolated from *B. megaterium* by the purified activator and depolymerase fractions of *R. rubrum*.

The hydrolase did not attack native PHB granules alone or in the presence of activator or trypsin which can substitute for activator (Merrick and Doudoroff, 1964). As previously mentioned special precautions had to be taken to completely free the hydrolase from depolymerase. Occasionally, however, purified preparations still contained a small amount of depolymerase. This contaminating enzyme could effectively be removed by a second fractionation of the hydroxylapatite eluate on DEAE-cellulose by the procedure previously described. This additional step does not significantly alter the final specific activity of the hydrolase.

Activator or depolymerase alone or in combination did not attack the dimer. None of the other esters examined, including purified PHB, were hydrolyzed at measurable rates.

Inhibitors. The enzyme appears to be quite resistant to sulfhydryl reagents. Less than 15% inactivation was observed after 20 min of preincubation of the enzyme with the following substances at 10^{-3} M: *p*-chloromercuribenzoate, *N*-ethylmaleimide, or iodoacetamide. The addition of sulfhydryl compounds, glutathione, mercaptoethanol, and cysteine was also without effect. The hydrolase is, however, very sensitive to DFP. After 20 min of preincubation of the enzyme with DFP at 1.8×10^{-5} and 1.7×10^{-6} M, the hydrolase was inhibited 84 and 48%, respectively.

The hydrolase was not stimulated by the addition of Ca^{2+}, Fe^{3+}, or Mn^{2+}. Prolonged dialysis of the enzyme against EDTA or direct addition of EDTA to reaction mixtures was without effect.

Discussion

Our previous investigations concerning the breakdown of PHB have utilized native PHB granules isolated from *B. megaterium* and the soluble depolymerizing enzyme system (activator and depolymerase) obtained from *R. rubrum*. One of the most interesting features of this system is the lability of native PHB granules. Native granules can be treated with a variety of chemical and physical agents which result in preparations that cannot be attacked by the soluble depolymerizing system (Merrick and Doudoroff, 1964). Examination of inactivated granules by electron microscopy has revealed that the treated granules no longer possess the distinct morphological features of native PHB granules (Merrick *et al.*, 1965). The morphological changes were mainly characterized by membrane fragmentation, loss of coalescence, and surface alterations, thus demonstrating that decreased susceptibility of the polymer to enzymatic hydrolysis is corrected with loss of morphological integrity. Furthermore, substances known to interfere with membrane structural organization also inhibit the depolymerizing process (Merrick, 1965). These data therefore suggested that the membrane or similar constituents of the PHB granules participate by unknown mechanisms in the depolymerization of the polymer.

The principal product of the digestion of native granules by the partially purified activator and depolymerase fractions was D(−)-β-hydroxybutyrate. However, a soluble ester, now identified as the dimeric ester of β-hydroxybutyrate, was also produced.

The data presented in this communication demonstrate that a hydrolase isolated from *R. rubrum* extracts converts the dimeric ester of β-hydroxybutyric acid to 2 equiv of the monomer. Presumably, the further metabolism of the monomer occurs by its oxidation to acetoacetate by a D(−) specific β-hydroxybutyrate:NAD-oxidoreductase (Shuster and Doudoroff, 1962). The hydrolase, like many other esterases, is susceptible to inhibition by DFP. However, unlike many other esterases it appears to be highly specific and only attacks oligomers of β-hydroxybutyrate. Other esters as well as β-hydroxybutyrate esterified with ethanol were not attacked. Its principal specificity is directed toward oligomers which possess the D(−) configuration but it also attacks 3-*O*-L-(3-D-hydroxybutyryl)hydroxybutyric acid at a low rate. In this respect the substrate stereospecificity of the *R. rubrum* hydrolase differs from that described for the *P. lemoignei* enzyme (Delafield *et al.*, 1965a). The latter enzyme in addition to the D(−) dimer attacks 3-*O*-D-(3-L-hydroxybutyryl)hydroxybutyric acid but not 3-*O*-L-(3-D-hydroxybutyryl)hydroxybutyric acid. Further specificity differences between the two enzymes were exhibited toward the presumed trimer which is hydrolyzed by the *P. lemoignei* enzyme but at low rates. The trimer is, however, a better substrate for the *R. rubrum* hydrolase than the dimer. This observation was somewhat surprising since esters of β-hydroxybutyrate other than dimer have not been detected as intermediates in the PHB-depolymerizing system. It is possible, however, that trimer or other low molecular weight oligomers of β-hydroxybutyrate may be the actual physiological substrate for the *R. rubrum* hydrolase. Thus it should be emphasized that the PHB-depolymerizing system consists of substrate PHB granules isolated from *B. megaterium* and purified depolymerizing factors from *R. rubrum*. For reasons presented in detail elsewhere (Merrick and Doudoroff, 1964), we believe that a study of this mixed system should provide a clearer understanding of the complex mechanisms which govern the breakdown of the lipid reserves of these bacteria. PHB granules isolated from *R. rubrum* undergo rapid self-digestion, and it has not as yet been possible to separate the PHB depolymerization enzymes from the substrate. However, extracts prepared from *R. rubrum* cells depleted of PHB readily attack native PHB granules from *B. megaterium* but not purified polymer. Isolated PHB granules from *B. megaterium* do not undergo appreciable self-digestion. It is therefore conceivable that the formation of dimer in the mixed system is a reflection of the substrate utilized. Further studies may reveal that in the breakdown of the self-digesting *R. rubrum* granules, low molecular weight oligomers but not dimer play an important role in the

depolymerization of the polymer. This possibility is currently being explored.

Acknowledgment

The authors wish to thank Miss Trudy Schwartz for her assistance in the purification and specificity studies on the dimer hydrolase.

References

Delafield, F. P., Cooksey, K. E., and Doudoroff, M. (1965a), *J. Biol. Chem. 240*, 4029.

Delafield, F. P., Doudoroff, M., Palleroni, N. J., Lusty, G. R., and Contopoulos, R. (1965b), *J. Bacteriol. 90*, 1455.

Hestrin, S. (1949), *J. Biol. Chem. 180*, 249.

Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Manganelli, R., and Brofazi, F. (1957), *Anal. Chem. 29*, 1441.

Merrick, J. M. (1965), *J. Bacteriol. 90*, 965.

Merrick, J. M., Delafield, F., and Doudoroff, M. (1962), *Federation Proc. 21*, 228.

Merrick, J. M., and Doudoroff, M. (1964), *J. Bacteriol. 88*, 60.

Merrick, J. M., Lundgren, D. G., and Pfister, R. M. (1965), *J. Bacteriol. 89*, 234.

Olsen, I., Merrick, J. M., and Goldstein, I. J. (1965), *Biochemistry 4*, 453.

Shuster, C. W., and Doudoroff, M. (1962), *J. Biol. Chem. 237*, 603.

Slepecky, R. A., and Law, J. H. (1960), *Anal. Chem. 32*, 1697.

Fructose Diphosphatase from Rabbit Liver. VII. Tyrosine Residues and Adenosine Monophosphate Inhibition*

S. Pontremoli, E. Grazi, and A. Accorsi

ABSTRACT: Treatment of crystalline rabbit liver fructose diphosphatase (FDPase) with *N*-acetylimidazole results in a time-dependent *O*-acetylation of ten tyrosyl residues.

In the first phase of the reaction two to three tyrosyl residues are acetylated with no change in catalytic properties. The acetylation of four additional residues is associated with loss of allosteric inhibition by adenosine monophosphate (AMP). Finally, with the acetylation of the last few residues the catalytic activity is abolished. The last phase is blocked by the substrate, fructose 1,6-diphosphate, while the inhibitor, AMP, protects the second group of tyrosyl residues. The evidence for acetylation of tyrosyl residues is based on spectral changes at 278 mμ and on the ability of hydroxylamine at pH 7.5 to reverse these effects. The characteristics of allosteric inhibition by AMP have been examined.

It is generally recognized that a critical and essentially irreversible step in gluconeogenesis is the conversion of fructose 1,6-diphosphate to fructose 6-phosphate, catalyzed by the enzyme FDPase.[1] Several lines of evidence point to a key role for this enzyme in the gluconeogenic sequence (Krebs *et al.*, 1964; Salas *et al.*, 1965; Fraenkel and Horecker, 1965). Evidence has also been obtained for the regulation of this enzyme, both *in vivo* (Weber *et al.*, 1965) and *in vitro* (Mangiarotti and Pontremoli, 1963; Pontremoli *et al.*, 1965a,b), by a number of biological compounds and chemical reagents.

An important regulatory mechanism (Taketa and Pogell, 1963; Newsholme, 1963) involves the specific and reversible allosteric inhibition of the enzyme by AMP. The authors have recently observed that treatment of crystalline rabbit liver FDPase with acetylimidazole leads to the acetylation of approximately ten tyrosyl residues with almost complete loss of catalytic activity which can be fully recovered by deacetylation with hydroxylamine at neutral pH (Pontremoli *et al.*, 1966).

However, kinetic analysis of the acetylation reaction has now shown that the partially acetylated enzyme, containing six *O*-acetyltyrosines, retains full catalytic activity but is almost completely desensitized against

* From the Istituto di Chimica Biologica, University of Ferrara, Ferrara, Italy. *Received July 15, 1966.* Supported by a grant from the Italian Consiglio Nazionale delle Ricerche, Impresa Enzimologia, by Grant GM 12291-02 from the National Institutes of Health, and by NATO Grant 218.

[1] Abbreviations used: FDPase, fructose diphosphatase; AMP, adenosine monophosphate; TPN^+, oxidized triphosphopyridine nucleotide; TPNH, reduced TPN.