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The Non-Involvement of Acyl-Carrier Protein in Poly-β-hydroxybutyric Acid Biosynthesis in *Azotobacter beijerinckii*

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The pathway of PHB† biosynthesis has not been fully investigated for any one genus of micro-organisms in which it is produced. Merrick & Doudoroff (1961) observed a small incorporation of radioactivity into PHB when crude extracts of *Rhodospirillum rubrum* were incubated with [1-^{14}C]-acetate, ATP, CoA and reduced nicotinamide coenzymes, but no direct activation of β-hydroxybutyric acid with ATP and CoA could be demonstrated. A particulate fraction isolated by these authors from either *R. rubrum* or *Bacillus megaterium* KM was found to incorporate radioactivity into PHB from D(−)-β-hydroxy[U-^{14}C]butyryl-CoA. The association of PHB synthetase with native granules isolated and purified from *B. megaterium* was confirmed by Merrick & Doudoroff (1964) and the enzyme later characterized by Griebel, Smith & Merrick (1968). A particulate membrane-bound PHB synthetase from *Azotobacter beijerinckii* has been characterized and, as for the *B. megaterium* enzyme, shown to possess a functional thiol group (Ritchie, 1968).

Recently the biosynthesis of saturated fatty acids in micro-organisms has been found to proceed via the ACP (Vagelos, Majerus, Alberts, Larrabee & Ailhaud, 1966). ACP is also involved in other biosynthetic reactions of lipid metabolism, e.g. unsaturated fatty acids (Nagai & Bloch, 1967), and a possible role for it in the biosynthesis of PHB could not be excluded. We have therefore tested this possibility.

An ACP was isolated in substrate quantities from *A. beijerinckii* and used for the chemical preparation of D(−)-β-hydroxy[U-^{14}C]butyryl-ACP. It was shown that no significant radioactivity was incorporated from the ACP ester into PHB under conditions that permitted ^{14}C incorporation from D(−)-β-hydroxy[U-^{14}C]butyryl-CoA by a preparation of native granules possessing PHB synthetase activity.

A. beijerinckii N.C.I.B. 9067 was grown at 30° with aeration in nitrogen-free medium containing (g./l.): $MgSO_4,7H_2O$, 0·2; $CaCl_2$, 0·005; $FeSO_4,7H_2O$, 0·006; $Na_2MoO_4,2H_2O$, 0·005; K_2HPO_4, 1·0; NaCl, 0·2; glucose, 10·0. ACP was isolated essentially by the method used by Goldman, Alberts & Vagelos (1963*a*,*b*) for *Escherichia coli*, with omission of chromatography on Sephadex G-75. It was purified to homogeneity.

Purified native granules with an active PHB synthetase were prepared from *A. beijerinckii* by a method similar to that of Merrick & Doudoroff (1964). Cells were suspended in 0·05M-KH_2PO_4–K_2HPO_4 buffer, pH 7·5, containing 0·01M-2-mercaptoethanol, and disrupted by two passages through a French pressure cell followed by ultrasonic treatment for 30sec. at 3·5A with a Soniprobe type 1130A (Dawe Instruments Ltd., London W.3). The extract was treated with deoxyribonuclease (1·5μg./ml.) for 10min. at 4°. The native granules were isolated by centrifuging the extract on a layer of glycerol in a swing-out rotor at 4100*g* for 20min. at 2° (MSE Mistral 6L, rotor no. 59549). After removal of the extract supernatant the PHB granules at the interface were resuspended in 0·05M-tris–HCl buffer, pH 8·0, containing 0·01M-2-mercaptoethanol. The centrifugation over glycerol was repeated four times. The final suspension was protected by addition of bovine serum albumin (fraction V; Sigma Chemical Co., St Louis, Mo., U.S.A.) at 5·0mg./ml. and dialysed against 0·05M-tris–HCl buffer, pH 8·0, containing 0·01M-2-mercaptoethanol.

D(−)-β-Hydroxy[U-^{14}C]butyric acid was prepared by alkaline hydrolysis of PHB granules from *A. beijerinckii* grown in the presence of D-[U-^{14}C]-glucose. The hydrolysate, a mixture of D(−)-β-hydroxybutyric acid and crotonic acid, was passed over an ion-exchange resin [Calbiochem AG-50W (X1; H^+ form)] and the free acids were concentrated in a rotary evaporator at 40°. The acids were separated on a silicic acid column by the method of Neish (1949).

The mixed anhydride of D(−)-β-hydroxy-[U-^{14}C]butyric acid and ethyl hydrogen carbonate was prepared by the method of Wieland & Rueff (1953). Transesterification with CoA was effected by the method of Simon & Shemin (1953). Yields were 90–100%, as measured by the hydroxamate

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† Abbreviations: PHB, poly-β-hydroxybutyric acid; ACP, acyl-carrier protein.

assay (Lipmann & Tuttle, 1945). Free D(−)-β-hydroxy[U-^{14}C]butyric acid was removed by ascending paper chromatography (Whatman 3MM) at 18° with the solvent system ethanol–0·1M-sodium acetate, pH 4·5 (1:1, v/v). The major ester band (R_F 0·6) was eluted with acidified water and assayed by hydroxamate formation.

The preparation of D(−)-β-hydroxy[U-^{14}C]-butyryl-ACP was analogous to the method of Alberts, Majerus, Talamo & Vagelos (1964). ACP (1·5μmoles, 16·5mg.) was dissolved in 2ml. of 0·01M-KH_2PO_4–K_2HPO_4 buffer, pH 6·3, containing 0·01M-2-mercaptoethanol, and left at 4° for 12hr. The 2-mercaptoethanol was removed on a column (1cm. × 4cm.) of DEAE-cellulose, previously equilibrated with 0·01M-KH_2PO_4–K_2HPO_4 buffer, pH 6·3, at room temperature, by washing with the phosphate buffer solution. Reduced ACP was eluted with the same buffer containing 0·5M-LiCl. The protein solution was adjusted to pH 8·0 with 0·2M-$KHCO_3$ and allowed to react for 45min. with 114μmoles of the mixed anhydride in a stream of O_2-free N_2 at 2°. The reaction mixture was acidified with 1·0M-HCl, when the ACP ester was precipitated. After centrifuging, the supernatant liquid was decanted and the ACP ester resuspended in 0·1M-HCl before recentrifuging. The washing procedure was repeated three times and the ACP ester finally dissolved in 1·0ml. of 0·1M-KH_2PO_4–K_2HPO_4 buffer, pH 8·0, and estimated by hydroxamate formation.

After incubation the reaction mixtures were centrifuged at 8900*g* for 2min. Samples (0·2ml.) of the supernatants were transferred to scintillation vials, dried and dissolved in 0·2ml. of Hyamine hydroxide (1·0M solution in methanol), and 10ml. of toluene scintillator solution was added. PHB granules were transferred quantitatively on to membrane filters (Sartorius MF12; 0·05μm., 2·5cm. diam.) with 6ml. of 0·01M-sodium DL-β-hydroxybutyrate carrier solution. Granules were washed on the filter with 4ml. of alkaline hypochlorite, pH 9·8 (Williamson & Wilkinson, 1958), and distilled water. The filters were dried with 0·5ml. of diethyl ether, transferred to scintillation vials and dried under vacuum over P_2O_5. The PHB was dissolved in 0·3ml. of chloroform, and 10ml. of toluene scintillator solution was added. All samples were counted in a Beckman LS-233 liquid-scintillation system. The recorded radioactivities (c.p.m.) for PHB samples were corrected for 4% loss of efficiency due to the presence of chloroform.

Table 1 records two experiments in which equivalent concentrations of each ester were incubated with native PHB granules. Of the radioactivity from D(−)-β-hydroxy[U-^{14}C]butyryl-CoA 20–30% was found in the polymer. Insignificant amounts of radioactivity were incorporated from D(−)-β-hydroxy[U-^{14}C]butyryl-ACP. A small amount of radioactivity remained in the supernatants of incubations with ACP ester, but most of the radioactivity was bound to, or adsorbed on, the denatured PHB granules and lost during extraction.

The high specific radioactivity associated with the ACP ester was probably a result of a side reaction between β-hydroxybutyryl residues and

Table 1. *PHB synthetase: incorporation of* ^{14}C *into PHB from* D(−)-β-*hydroxy*[U-^{14}C]*butyryl esters of CoA and ACP*

Each incubation mixture (0·5ml.) contained 10μmoles of tris–HCl buffer, pH 8·0, 0·25μmole of dithiothreitol and native PHB granule preparation (Expt. 1: 42μg. of PHB and 6·5μg. of protein/assay; Expt. 2: 44μg. of PHB and 7·0μg. of protein/assay); substrate was added after preincubation for 5min. at 30°. The reaction was terminated after 5min. by addition of 0·05ml. of 10% (w/v) $HClO_4$. The recorded radioactivity is the average of two 100min. counts. All counts were corrected for background by using control incubations without radioactive substrate.

Substrate	Expt. no.	Radioactivity (c.p.m./assay) Supernatant	PHB	Percentage incorporation into PHB	Percentage recovery of radioactivity
D(−)-β-Hydroxy[U-^{14}C]butyryl-CoA (0·12μmole, 864c.p.m.)	1	482	174	20·0	76·0
		522	186	22·0	82·0
		482	172	20·0	76·0
D(−)-β-Hydroxy[U-^{14}C]butyryl-CoA (0·036μmole, 252c.p.m.)	2	129	59	23·0	75·0
		121	91	36·0	84·0
		110	77	31·0	75·0
D(−)-β-Hydroxy[U-^{14}C]butyryl-ACP (0·10μmole, 3160c.p.m.)	1	55	21	0·7	2·4
		39	0	0·0	1·2
		72	5	0·2	2·4
D(−)-β-Hydroxy[U-^{14}C]butyryl-ACP (0·12μmole, 2910c.p.m.)	2	159	7	0·2	5·7
		165	7	0·2	5·9

amino acids other than the prosthetic group of ACP. Such effects have been reported during the chemical acylation of ACP from *Escherichia coli* with mixed fatty acyl anhydrides (Nagai & Bloch, 1967). These ACP esters were still active in enzymic systems.

A possible explanation for the non-utilization of the ACP ester in the biosynthesis of PHB, applicable to conditions both *in vitro* and *in vivo*, is adsorption of the protein moiety of the substrate on the granule surface effectively masking the active sites of the PHB synthetase.

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