A Preparation of D(–)-β-Hydroxybutyric Acid

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The D(–)-isomer of β-hydroxybutyric acid, which occurs in animal blood, is not readily obtainable. The methods of preparation hitherto used are isolation from the urine of an uncontrolled diabetic (Magnus-Levy, 1901) or fractional crystallization of alkaloid salts (Lehninger & Greville, 1953).

Many bacilli, notably *Bacillus megaterium* and *Bacillus cereus*, form granules of poly-β-hydroxybutyric acid when they are grown in a medium containing an adequate energy source (Macrae & Wilkinson, 1958). In favourable conditions the polymer makes up as much as 40% of the dry weight of the cells. The granules may be liberated by lysis of the cells with alkaline hypochlorite, and the polymer purified by solution in chloroform and precipitation with ether (Williamson & Wilkinson, 1958). The material has been shown to be a polymer of D(–)-β-hydroxybutyric acid (Péaud-Lenoël, 1949), but it is extremely resistant to acid hydrolysis, and during hydrolysis by alkali, which is very slow, considerable dehydration to crotonic acid takes place.

Hydrazinolysis has been used for splitting esters which are resistant to acid hydrolysis (Reed, 1957), and the use of this reagent is now reported for the preparation of pure D(–)-β-hydroxybutyric acid hydrazide, the free acid and its sodium salt. No racemization took place during any of the preparative procedures, nor was dehydration to crotonic acid observed.

MATERIALS AND METHODS

Preparation of poly-β-hydroxybutyric acid. *Bacillus cereus*, strain AC1, was grown in a medium containing (g./100 ml. of water): Na₂HPO₄, 0.6; KH₂PO₄, 0.3; NaCl, 0.3; NH₄Cl, 0.1; MgCl₂·6H₂O, 0.01; MnCl₂·4H₂O, 0.001; Casamino acids (Difco), 0.01; sodium acetate, 0.8. Glucose was sterilized separately and added to a final concentration of 0.1% (w/v). A portion (150 ml.) of medium was placed in a Roux bottle, which after inoculation was incubated at 37° for 48 hr. In some preparations *B. megaterium* grown by continuous culture was used.

The poly-β-hydroxybutyric acid was obtained from the cells essentially by the method of Williamson & Wilkinson (1958). The cells were suspended in the minimum volume of water and lysed by addition of 4–5 vol. of 15% sodium hypochlorite, adjusted to pH 10 with acid, and stirring for a few hours. The insoluble material was centrifuged off and washed several times with water. Sodium dithionite was

added if necessary to remove traces of chlorine. The precipitate was washed with acetone and then with ether, and then dissolved in chloroform. Insoluble material was removed by filtration. Poly-β-hydroxybutyric acid was precipitated from the chloroform solution by cooling to –20° and adding 1 vol. of ether.

Anhydrous hydrazine. This was prepared from hydrazine hydrate by distillation, first from solid NaOH and then from CaO or BaO.

Hydrazinolysis. Poly-β-hydroxybutyric acid (6 g.) was suspended in 15 ml. of anhydrous hydrazine in a conical flask closed with a glass bubble, and incubated at 37° for 18 hr., or at a higher temperature for a correspondingly shorter period. The surface area of the solvent should be large, because a certain amount of gas (presumably N₂) is evolved, which in a narrow vessel may separate the light polymer from the liquid. At 37°, dissolution of all but a small coloured residue appeared to be complete after 4 hr.

Absolutely anhydrous hydrazine was not necessary; depolymerization was found to occur with aqueous 70% (v/v) hydrazine solutions, but only after refluxing for several hours.

The solution was diluted with water and filtered to remove the insoluble residue (0.6 g.). Excess of hydrazine was removed by evaporating to dryness *in vacuo*, dissolving the crystals in water and evaporating to dryness again. The product was removed from the flask by dissolving in a few millilitres of boiling propan-1-ol, and allowed to crystallize. Recrystallization from propanol gave white needle-shaped crystals of D(–)-β-hydroxybutyric acid hydrazide (4.0 g.; yield 50%). The compound is very soluble in water and hot ethanol, moderately soluble in cold ethanol and insoluble in ether; m.p. 126–128° (uncorr.), [α]_D –30.0° (Found: C, 41.1; H, 8.3; N, 24.0; O, 26.6. Calc. for C₄H₁₀N₂O₂: C, 40.7; H, 8.5; N, 23.7; O, 27.1%).

The free acid could be obtained in rather poor yield by oxidation with the theoretical quantity of K₂Cr₂O₇ in N-H₂SO₄, followed by ether extraction. A better procedure was hydrolysis by refluxing for 3 hr. with 3N-HCl, which gave a solution with 93% of the optical rotation expected for the same quantity of free β-hydroxybutyric acid. The solution was evaporated to dryness twice to remove excess of HCl, and the resulting oil was extracted several times with ether. The extract was dried with anhydrous Na₂SO₄ and the ether evaporated to leave an oil, which has been reported (McKenzie, 1902) to crystallize only after standing for some months in a vacuum desiccator. A solution of the oil gave a single spot on paper chromatography [solvent ethanol-ammoniacal (NH₄)₂CO₃, 7:3, v/v (Hartley & Lawson, 1960)] and a single peak on chromatography on a silicic acid column (Bulen, Varner & Burrell, 1952).

From a portion of the ether extract of β-hydroxybutyric acid, the sodium salt was prepared by adding an equal volume of water and titrating to pH 7 with carbonate-free

NaOH. The water layer was separated and evaporated to dryness in a current of warm air. The resulting solid was recrystallized from ethanol, giving a 20% yield of the ethanol compound (Found: Na, 13.5. Calc. for $C_4H_7O_2Na$, C_4H_5OH : Na, 13.4%). $[\alpha]_D -14.6^\circ$ [expected, -14.4° ; Lehniger & Greville (1953), who also quote earlier authors]. The ethanol could be removed by dissolving the crystals in water, evaporating and finally drying in a vacuum desiccator.

DISCUSSION

The main loss in the preparative procedure appears to occur during the hydrazinolysis. The conversion into sodium β -hydroxybutyrate gave a poor yield simply because no attempt was made to retrieve the salt from the mother liquor after recrystallization from ethanol. Recrystallization is not really necessary, since the solid obtained by evaporating the aqueous solution after neutralization with sodium hydroxide appears to be pure. The loss in hydrazinolysis is partly apparent, since the granules of polymer contain 5–10% of lipid (Macrae & Wilkinson, 1958). Nevertheless, the recovery was below that expected. If free β -hydroxybutyric acid were produced during the depolymerization, e.g. from the $-CO_2H$ terminal groups of the chains, it, or its hydrazine salt, might have been lost during crystallization from propanol. This possibility has not been investigated. There might also have been a loss through some chemical reaction associated with the evolution of gas during depolymerization. It is presumed that this gas is nitrogen, arising from oxidation of hydrazine, although it is not immediately apparent what the oxidant can be.

It is of interest that poly- β -hydroxybutyrate should contain the D(–) isomer of the acid, if it is

formed by condensation of β -hydroxybutyryl residues from the CoA derivative. It has been shown that in mammalian liver the metabolically active form is L(+)- β -hydroxybutyryl-CoA (Lehniger & Greville, 1953; Lynen & Decker, 1957).

SUMMARY

1. Pure D(–)- β -hydroxybutyric acid has been prepared by the hydrazinolysis of bacterial poly- β -hydroxybutyric acid.

2. The main product of hydrazinolysis, D(–)- β -hydroxybutyric hydrazide, was obtained in crystalline form and was converted into the free acid by hydrolysis with hydrochloric acid at 105° for 3 hr.

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The Behaviour of the *Bz*-Methylindoles as Substrates and Inhibitors for *Neurospora crassa* Tryptophan Synthase

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The present investigation was undertaken to gain further knowledge of the nature of enzyme–substrate interaction in tryptophan synthesis and complement a previous investigation of the degradation of certain *Bz*-substituted tryptophans by *Escherichia coli* tryptophanase (Hall, Leeson, Rydon & Tweddle, 1960).

Tryptophan synthase [L-serine hydro-lyase (adding indole), EC 4.2.1.20] was first recognized by Tatum & Bonner (1943, 1944), who demonstrated the ability of extracts of *Neurospora crassa* to catalyse a condensation between indole and L-serine to yield L-tryptophan. The requirement of the enzyme for pyridoxal phosphate was demonstrated