Purification and Properties of Crystalline 3-Hydroxybutyrate Dehydrogenase from *Rhodopseudomonas spheroides*

By H. U. BERGMEYER, K. GAWEHN and H. KLOTZSCH
C. F. Boehringer und Soehne G.m.b.H., Tutzing, Germany
and H. A. KREBS and D. H. WILLIAMSON
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

(*Received* 22 *July* 1966)

1. The purification and crystallization of 3-hydroxybutyrate dehydrogenase from extracts of *Rhodopseudomonas spheroides* is described. 2. The molecular weight was calculated to be 85 000 by sedimentation equilibrium. 3. Although the enzyme is stable at 0–4°, dilute solutions are rapidly inactivated at 37°; $NADH_2$ or Ca^{2+} ions prevent this inactivation. 4. The enzyme is extremely sensitive to mercurials, but can be protected by $NADH_2$ or Ca^{2+} ions. 5. From studies on *p*-hydroxymercuribenzoate binding it is estimated that the enzyme contains 5–6 moles of rapidly reacting thiol groups/mole. 6. D-Lactate and DL-2-hydroxybutyrate are competitive inhibitors of D-3-hydroxybutyrate oxidation. 7. The properties of the crystalline enzyme are compared with those of 3-hydroxybutyrate dehydrogenase preparations from other sources.

Mammalian 3-hydroxybutyrate dehydrogenase (D-3-hydroxybutyrate–NAD oxidoreductase, EC 1.1.1.30) is associated with the insoluble, particulate fractions of the cell, and has only been solubilized with great difficulty (Sekuzu, Jurtshuk & Green 1963). In contrast, the 3-hydroxybutyrate dehydrogenase that occurs in certain species of microorganisms can be easily solubilized, is relatively stable and has been partially purified (Shuster & Doudoroff, 1962; Williamson, Mellanby & Krebs, 1962). The enzyme is of special interest because the reaction it catalyses forms the basis of a rapid and specific method for the determination of the ketone bodies, acetoacetate and D-3-hydroxybutyrate (Williamson *et al.* 1962; Bergmeyer & Bernt, 1965).

The present paper describes the purification and crystallization of 3-hydroxybutyrate dehydrogenase from extracts of *Rhodopseudomonas spheroides*, and some of its relevant properties including molecular weight.

MATERIALS AND METHODS

Chemicals. The lithium salts of acetoacetic acid, 3-oxopentanoic acid and 3-oxohexanoic acid were prepared by the hydrolysis of the corresponding ethyl esters with lithium hydroxide according to the procedure described for acetoacetic acid by Hall (1962). The 3-oxo acid content of the lithium salts was determined manometrically by decarboxylation with aniline citrate (Edson, 1935). DL-3-hydroxypentanoic acid was prepared by the Reformatsky reaction between propionaldehyde and ethyl bromoacetate in the presence of zinc. Similarly, 3-hydroxyhexanoic acid was prepared by the condensation of butyraldehyde and ethyl bromoacetate. The method used for the preparation of these 3-hydroxy acids and their isolation as the sodium salts was as described by Adickes & Andresen (1944). L-Lactic acid was prepared by the method of Krebs (1961).

Sodium DL-3-hydroxybutyrate, sodium glycollate, lithium DL-lactate and laevulic acid were obtained from British Drug Houses Ltd., Poole, Dorset. The sample of sodium DL-3-hydroxybutyrate used in this work analysed 98–100% pure by the enzymic method of Williamson *et al.* (1962) assuming that it contained 1 mol. of water/mol. Unless otherwise stated the term 3-hydroxybutyrate refers to this material, but all concentrations given here refer to the D-isomer only.

Ethyl 3-oxopentanoate, ethyl 3-oxohexanoate, 2-oxobutyric acid, 2-oxopentanoic acid, 2-hydroxybutyric acid, 2-hydroxypentanoic acid and 2-aminobutyric acid were obtained from Fluka A.-G., Buchs SG, Switzerland.

The nicotinamide–adenine dinucleotides (NAD, $NADH_2$, NADP and $NADPH_2$) and certain analogues (acetylpyridine and thionicotinamide) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Solutions of the coenzymes were prepared freshly each day.

Preparation of DEAE-Sephadex. DEAE-Sephadex A-50 (500 g., pearl form) (Pharmacia, Uppsala, Sweden) was suspended in 20 l. of 0·1 M-NaCl, allowed to swell overnight and then filtered by suction on a Buchner funnel. Approximately 7 l. of N-NaOH was poured on to the resin and after about 2 hr. the fluid was sucked off. The Sephadex was then suspended in 10 l. of distilled water and neutralized with 0·2 M-KH_2PO_4. The resin was collected by suction and washed three times with about 10 l. of 0·2 M-phosphate

buffer, pH 7·6, each time; the filter cake was suspended in the buffer and allowed to stand for 10–15 min. Finally, the Sephadex was washed and equilibrated three times with 10 l. portions of 0·05 M-phosphate buffer, pH 7·6, or distilled water respectively. The equilibrated DEAE-Sephadex was stored at 4° as a compressed moist cake.

Determination of 3-hydroxybutyrate-dehydrogenase activity. The optimum conditions for the measurement of 3-hydroxybutyrate-dehydrogenase activity were found to be as follows: (*a*) for the oxidation reaction: tris buffer, pH 8·4 (33 mM), NAD (1·8 mM) and 3-hydroxybutyrate (11 mM); (*b*) for the reduction reaction: phosphate buffer, pH 6·8 (33 mM), $NADH_2$ (0·33 mM) and acetoacetate (4 mM). All measurements were carried out at 25° in either a Unicam SP.500 or an Eppendorf photometer (Netheler und Hinz, Hamburg, Germany). The enzyme was diluted where necessary with cold 0·02 M-phosphate buffer, pH 6·8. One unit of enzyme activity is defined as the amount of enzyme which catalyses the conversion of 1 μmole of substrate (acetoacetate or 3-hydroxybutyrate)/min. at 25°. The specific activity is expressed in units/mg. of protein. The protein was determined by a modified biuret method (Beisenherz *et al.* 1953).

Determination of ammonium sulphate concentration. Ammonium sulphate was determined by a titrimetric method with Alizarin S as indicator (Bergmeyer, Holz, Kauder, Möllering & Wieland, 1961). The concentration is expressed in moles/l.

EXPERIMENTAL

Purification and crystallization

Extraction of enzyme. Freeze-dried *Rhodopseudomonas spheroides* (500 g.), which had been grown under conditions similar to those described by Williamson *et al.* (1962), was suspended in 10 l. of distilled water, cooled to 3–5° and the cells were disintegrated in a high-pressure laboratory homogenizer (Manton-Gaulin S.A., Boston 49, Mass., U.S.A.) at about 400 atm. After circulation through the machine the suspension was immediately cooled again. When the operation was complete the machine was rinsed through with distilled water. Approx. 13 l. of suspension was obtained and this was centrifuged at 13 500 *g* in a Sharples centrifuge. The clear supernatant fluid was cooled to about 0°.

Protamine sulphate precipitation. To the supernatant fluid was added, with stirring, 0·1 vol. of protamine sulphate solution (20 mg./ml.; pH 7). The precipitate was centrifuged off at 4500 *g* for 10 min. and discarded.

First DEAE-Sephadex absorption. The clear supernatant fluid after protamine sulphate treatment was diluted with 0·5 vol. of distilled water, and 1 vol. of DEAE-Sephadex equilibrated with water (see the Materials and Methods section) was stirred in. After standing for 15 min., the Sephadex was collected on a Buchner funnel. At least 95% of the 3-hydroxybutyrate-dehydrogenase activity should be adsorbed on to the resin. The filter cake was washed twice with about 10 l. of 0·2 M-phosphate buffer, pH 7·6, and once with a solution of 0·1 M-phosphate buffer, pH 7·6, containing 0·2 M-NaCl; the resin was suspended in the solution and then collected on a Buchner funnel by suction. The enzyme was eluted in a similar manner with about 7 l. of 0·2 M-phosphate buffer, pH 7·6, containing 0·4 M-NaCl. If the elution was not complete, the NaCl concentration was increased to 0·8 M.

Ammonium sulphate fractionation. Solid ammonium sulphate was added to the combined eluates (about 20 l.) to give a final concentration of 2·4 M. The precipitate was collected by centrifugation at 4500 *g* for 2 hr., dissolved in about 1·5 l. of distilled water and any insoluble material removed by centrifugation at 14 000 *g* for 30 min. Ammonium sulphate was added to the clear supernatant fluid to give a final concentration of 1·35 M. The inactive precipitate was centrifuged off at 14 000 *g* for 30 min. and discarded, and the supernatant fluid brought to 2·4 M by further addition of solid ammonium sulphate. The active precipitate was centrifuged off at 14 000 *g* for 30 min., dissolved in distilled water and then dialysed for 2 hr. against running tap water.

Second DEAE-Sephadex absorption. The dialysed enzyme solution (about 1·6 l.) was treated with DEAE-Sephadex as described above except that in this case resin equilibrated with 0·05 M-phosphate buffer, pH 7·6, was used. The enzyme was eluted off again with about 700 ml. of 0·2 M-phosphate buffer, pH 7·6, containing NaCl: 0·1 M for the first elution, 0·2 M for the second and third, and 0·4 M for the fourth. The eluates were collected separately, the most active were combined and brought to 2·4 M by addition of solid ammonium sulphate. The precipitate was centrifuged off (30 min. at 14 000 *g*), dissolved in distilled water and dialysed against running tap water for 2 hr.

First chromatography on DEAE-Sephadex. The dialysate was adsorbed on to a DEAE-Sephadex column (5 cm. × 50 cm.) and the column was washed with 2–3 bed volumes of 0·05 M-phosphate buffer, pH 7·6, containing 0·20 M-NaCl. The enzyme was then eluted with about 800 ml. of 0·05 M-phosphate buffer, pH 7·6, containing 0·25 M- or 0·3 M-NaCl. The most active fractions were combined and solid am-

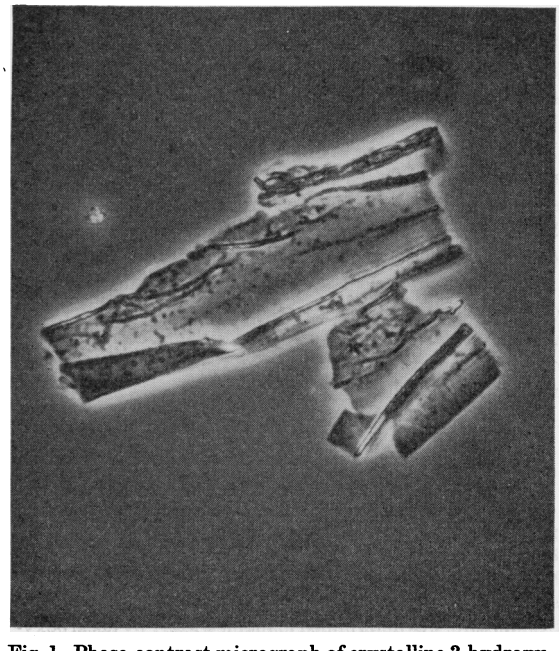

Fig. 1. Phase-contrast micrograph of crystalline 3-hydroxybutyrate dehydrogenase. Magnification × 440.

Table 1. *Purification and crystallization of 3-hydroxybutyrate dehydrogenase*

Summary of a typical purification of the dehydrogenase from 500 g. of freeze-dried *Rhodopseudomonas spheroides*. Only the most active fractions were taken at steps (6) and (7).

Step	Total volume (ml.)	Total activity (units)	Total protein (mg.)	Specific activity (units/mg.)	Yield (%)
(1) Extract	13 000	33 000	300 000	0·11	100
(2) First DEAE-Sephadex absorption	20 000	23 000	41 000	0·56	70
(3) Ammonium sulphate fractionation	710	20 000	29 000	0·69	60
(4) Second DEAE-Sephadex absorption	460	16 000	13 000	1·23	49
(5) First chromatography on DEAE-Sephadex	700	11 500	2 500	4·6	35
(6) Second chromatography on DEAE-Sephadex	440	6 000	800	7·5	18
(7) Third chromatography on DEAE-Sephadex	210	3 500	210	16·7	11
(8) Crystals	35	3 100	180	17·2	9

monium sulphate was added to give a final concentration of 2·4 M. The precipitate was centrifuged off at 14 000 *g* for 30 min., dissolved in a little distilled water and dialysed against running tap water for 2 hr.

Second chromatography on DEAE-Sephadex. The dialysed enzyme solution was rechromatographed as described above. The most active fractions were combined, cooled to 0° and brought to 1·7 M with respect to ammonium sulphate. The inactive precipitate was removed by centrifugation and solid ammonium sulphate was slowly added to the clear supernatant at 0° to give a final concentration of 2·4 M. The precipitate was collected by centrifugation at 14 000 *g*, dissolved in distilled water and dialysed as described above.

Crystallization. The dialysed enzyme solution was rechromatographed as described above. The most active fractions were combined and solid ammonium sulphate was added very slowly to give a final concentration of 2·4 M; the pH was adjusted to 7·0 with dilute ammonia. As soon as turbidity appeared in the solution, the ammonium sulphate was added more slowly with constant stirring. The enzyme crystallized in very fine plates (Fig. 1).

The result of a typical isolation and crystallization is shown in Table 1.

RESULTS

Properties of the crystalline enzyme

Molecular weight. The Spinco model E ultracentrifuge was used for the measurements. The molecular weight was determined by a sedimentation-equilibrium method (Yphantis, 1960) employing a short-column multi-channel cell. The rotor speed was 12 590 rev./min. at 20°. The protein was dissolved in 0·02 M-phosphate buffer, pH 7·4, and the concentration was 0·81% (w/v). The partial specific volume was assumed to be 0·75 and the average molecular weight was found to be 85 000 ± 8% (four pictures).

Optimum pH. Acetate, phosphate, tris–hydrochloric acid and glycine–sodium hydroxide buffers were used for the determination of the pH optimum of the oxidation and reduction reactions. None of these buffers appeared to inhibit the dehydrogenase appreciably, since assays carried out with different buffers of the same pH gave similar values for the enzyme activity. The reduction reaction with acetoacetate as substrate exhibited optimum activity between pH 6·2 and 6·9; the activity declined slowly in the alkaline range to give 50% of the maximum activity at pH 9·4 (Fig. 2*b*). The oxidation reaction (3-hydroxybutyrate and NAD) showed a broader range of optimum activity (between pH 7·0 and 9·0), which fell off rapidly above pH 9·5 and below pH 6·5 (Fig. 2*a*). However, it is difficult to obtain accurate values for the rate of NAD reduction at low pH values because of the rapid attainment of the equilibrium of the dehydrogenase system.

Specificity. The enzyme did not react with L(+)-3-hydroxybutyrate even when this was present at a concentration of 50 mM. Of other 3-hydroxy acids tested, 3-hydroxypropionate did not react, whereas 3-hydroxypentanoate and 3-hydroxyhexanoate were oxidized about 20 times more slowly than 3-hydroxybutyrate (Table 2). 3-Oxopentanoate and 3-oxohexanoate oxidized $NADH_2$ in the presence of the enzyme, although the rate was considerably lower than with acetoacetate (Table 2). NADP and $NADPH_2$ cannot replace NAD and $NADH_2$ in the oxidation and reduction reactions respectively. 3-Acetylpyridine–adenine dinucleotide and thionicotinamide–adenine dinucleotide reacted at about one-tenth of the rate with NAD.

Michaelis constants. The apparent Michaelis constants, K_m, for acetoacetate, 3-oxopentanoate and 3-oxohexanoate were found to be 0·28 mM, 0·031 mM and 0·54 mM respectively at pH 7·4 (with 0·2 mM-$NADH_2$) by the method of Lineweaver & Burk (1934). High concentrations of acetoacetate inhibited the enzyme (about 50% at 30 mM), whereas 3-oxohexanoate did not inhibit at a concentration (260 mM), 40 times that required to saturate the enzyme. A K_m value of 0·41 mM was obtained

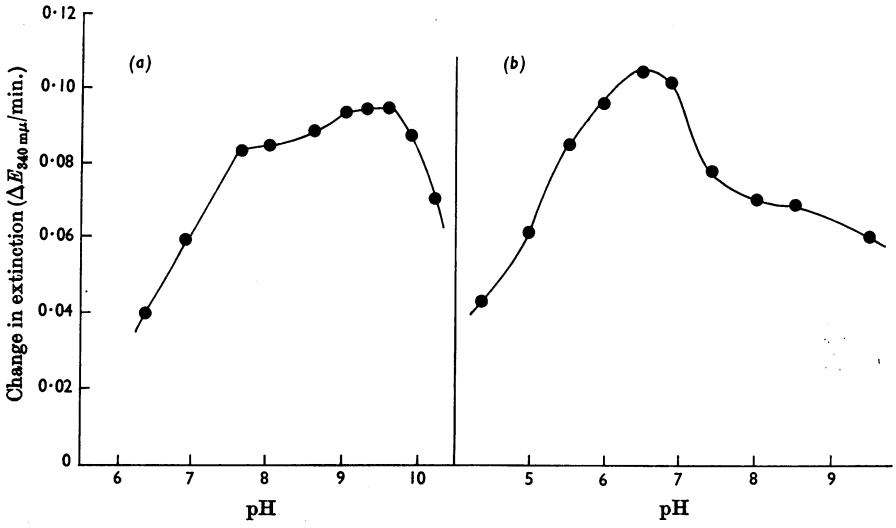

Fig. 2. Effect of pH on 3-hydroxybutyrate-dehydrogenase activity. The composition of the reaction mixtures was described in the Materials and Methods section and the amount of protein taken was about 3μg. (*a*) Hydroxybutyrate oxidation; (*b*) acetoacetate reduction.

Table 2. *Comparative activity of 3-hydroxybutyrate dehydrogenase with various homologues of acetoacetate and 3-hydroxybutyrate*

For the oxidation reaction the assay system consisted of: 100μmoles of tris–HCl buffer, pH8·5, 10μmoles of 3-hydroxy acid (sodium salt), 1 μmole of NAD and distilled water to 3·1ml. For the reduction reaction 100μmoles of tris–HCl buffer, pH 7·0, 10μmoles of 3-oxo acid (sodium salt), 0·5μmole of $NADH_2$ and distilled water to 3·1ml. The reaction was started by addition of enzyme (approx. 2·3μg. of protein).

Compound	Change in extinction ($\Delta E_{340\,m\mu}$/min.)	Comparative activity (%)
Oxidation reaction		
3-Hydroxybutyrate	0·080	100
3-Hydroxypropionate	<0·0003	<0·3
3-Hydroxypentanoate	0·004	5
3-Hydroxyhexanoate	0·003	4
Reduction reaction		
Acetoacetate	0·075	100
3-Oxopentanoate	0·004	5
3-Oxohexanoate	0·003	4

for 3-hydroxybutyrate at pH 8·5 (0·33mM-NAD). The K_m for $NADH_2$ was calculated to be 0·054mM at pH 7·0 and with 3·3mM-acetoacetate, whereas that for NAD was 0·080mM at pH 8·5 (3·3mM-hydroxybutyrate).

Stability. During the purification of the dehydrogenase it was noted that, although dilute solutions of the enzyme were stable at 0–4° for several days, on warming to 37° the enzyme rapidly lost activity. This inactivation was dependent on the protein concentration; dehydrogenase preparations containing more than 5mg. of protein/ml. showed no significant loss of activity after incubation for 1hr. at 37°. The inactivation of 3-hydroxybutyrate dehydrogenase at low protein concentrations seemed to be a specific effect on this enzyme because malate-dehydrogenase activity contained in crude preparations was not altered under conditions where all the 3-hydroxybutyrate dehydrogenase activity was lost. The influence of a number of

Table 3. *Effect of nicotinamide–adenine dinucleotides on the stability of 3-hydroxybutyrate dehydrogenase at 37°*

The incubation mixture consisted of: 0·3μmole of the particular nucleotide, enzyme (23μg. of protein) and 10mM-phosphate buffer, pH 7·0, to a final volume of 0·5ml. Samples were removed at 0, 5, 15 and 30min. for determination of enzyme activity. The results are expressed as a percentage of the initial activity at 0min. (100%).

		Activity (% of initial activity)		
Addition	Time	5min.	15min.	30min.
None		49	33	10
NAD		100	87	54
$NADH_2$		100	100	100
NADP		59	35	13
$NADPH_2$		63	47	19

potential protective agents on the stability of the enzyme in dilute solution at 37° was tested. Incubation on the enzyme (45 μg./ml.) in the presence of its substrates, acetoacetate and 3-hydroxybutyrate (3·3 mM), did not result in any significant stabilization of the enzyme. The presence of cysteine or mercaptoethanol (1 mM) or incubation under anaerobic conditions (nitrogen) were without effect. $NADH_2$ exerted a marked protective action on the enzyme, but other nicotinamide–adenine dinucleotides were much less effective (Table 3). Of the cations tested, the protection afforded by Ca^{2+} was comparable with that by $NADH_2$; Mn^{2+} and Mg^{2+} protected the enzyme to a small extent; Zn^{2+} and Na^+ did not protect at all (Table 4). The protective action of $NADH_2$ and Ca^{2+} was observed with enzyme preparations of varying purity.

Inhibitor studies with thiol reagents. The enzyme was extremely sensitive to a number of thiol inhibitors of the mercurial type (Table 5). In these experiments the enzyme was preincubated with the thiol reagent and the assay of dehydrogenase activity was started by addition of the other components of the system (acetoacetate and $NADH_2$). When the mercurial was added last to the cuvette no inhibition of the enzyme occurred. This suggested that a component of the assay system protected the enzyme from the action of the inhibitor. Accordingly, the enzyme was preincubated with excess of chloromercuribenzene-*p*-sulphonate and the individual reactants of the dehydrogenase assay system in turn. Only $NADH_2$ was found to protect the enzyme under these conditions (Table 6). Similar results were obtained with the other mercurial compounds and $NADH_2$.

In view of the increased stability of the enzyme at 37° in the presence of bivalent cations, particularly Ca^{2+}, the possibility that these might also protect the enzyme against inactivation by thiol reagents was tested. The dehydrogenase was protected by Ca^{2+} from inactivation by chloromercuribenzene-*p*-sulphonate, but Mn^{2+} and Mg^{2+} afforded virtually no protection (Table 6).

Table 4. *Effect of various cations on the stability of 3-hydroxybutyrate dehydrogenase at* 37°

The incubation mixture consisted of: 0·5 μmole of inorganic salt, enzyme (23 μg. of protein) and 10 mM-phosphate buffer, pH 7·0, to a final volume of 0·5 ml. Samples were removed at 0, 5, 15 and 30 min. for determination of enzyme activity. The results are expressed as a percentage of the initial activity at 0 min. (100%).

Addition	Activity (% of intial activity) Time ... ... 5 min.	15 min.	30 min.
None	59	38	16
NaCl	58	35	15
$MgCl_2$	64	47	28
$MnCl_2$	93	81	72
$CaCl_2$	100	100	100
$ZnCl_2$	57	36	14

Table 5. *Effects of thiol reagents on the activity of 3-hydroxybutyrate dehydrogenase*

The enzyme preparation (approx. 1 μg. protein) was preincubated for 5 min. at 25° in a cuvette with 100 μmoles of phosphate buffer, pH 7·0, and the respective thiol reagent in a final volume of 3·0 ml. $NADH_2$ (0·5 μmole) and acetoacetate (10 μmoles) were then added and the assay of activity was carried out in the usual way. The results are expressed as the percentage inhibition compared with a control preincubated without thiol reagent.

Additions	Final concn. (μm.)	Inhibition (%)
p-Hydroxymercuribenzoate	0·33	76
	0·66	85
	1·0	100
Chloromercuribenzene-*p*-sulphonate	0·1	76
	0·33	90
	1·0	100
Mercuric acetate	0·1	65
	0·33	96
	1·0	100
Iodoacetate	1000	0

Table 6. *Protection of 3-hydroxybutyrate dehydrogenase against inactivation by chloromercuribenzene-*p*-sulphonate*

The enzyme (approx. 1 μg. of protein) was preincubated for 5 min. at 25° in a cuvette with chloromercuribenzene-*p*-sulphonate (final concn. 3·3 μM), 100 μmoles of phosphate buffer, pH 7·0, and the protective agent to be tested in a final volume of 3·0 ml. The determination of activity was carried out by the addition of $NADH_2$ and acetoacetate. The results are expressed as the percentage inhibition compared with a control preincubated without mercurial.

Additions	Final concn. (mM)	Inhibition (%)
None	—	100
NAD	0·4	100
$NADH_2$	0·03	90
$NADH_2$	0·05	64
$NADH_2$	0·07	46
$NADH_2$	0·10	34
NADP	0·4	100
$NADPH_2$	0·2	100
Acetoacetate	3·3	100
Hydroxybutyrate	3·3	100
$CaCl_2$	1·0	25
$CaCl_2$	0·33	62
$MgCl_2$	1·0	95
$MnCl_2$	1·0	85

Table 7. *Effect of $NADH_2$ on the inhibition of 3-hydroxybutyrate dehydrogenase by p-hydroxymercuribenzoate*

Cuvettes contained in a final volume of 1·0 ml.: (*a*) 50 μmoles of tris–HCl buffer, pH 7·4, and 4 mμmoles (350 μg.) of hydroxybutyrate dehydrogenase; (*b*) as for (*a*) plus 30 mμmoles of $NADH_2$. The cuvettes were incubated at 25° and 10 μl. samples were removed for assay of enzyme activity (zero time). *p*-Hydroxymercuribenzoate (10 mμmoles) was added and mercaptide formation followed at 255 mμ (Boyer, 1954). Samples of the mixtures were taken for determination of enzyme activity when no further increase in extinction at 255 mμ occurred. Further additions of mercurial were made and the process was repeated.

p-Hydroxymercuribenzoate added (mμmoles)	Time (min.)	Enzyme activity (%) Cuvette (*a*)	Cuvette (*b*)
None	0	100	100
10	3	48	90
10	6	7	60
10	9	2	30

To obtain information on the reactivity and approximate number of thiol groups combining with *p*-hydroxymercuribenzoate, mercaptide formation was measured spectrophotometrically by the method of Boyer (1954). The sensitive thiol groups reacted rapidly with the mercurial (within 10 min.) and it was calculated from the change in extinction at 255 mμ that the enzyme contained 5–6 moles of rapidly reacting thiol groups/mole of protein. Under similar conditions the presence of $NADH_2$ resulted in appreciable protection of the catalytic activity of the enzyme (Table 7) although the amount of mercurial bound to the enzyme (as judged from the extinction changes at 255 mμ) was not significantly different. The inclusion of Ca^{2+} ions in the incubation mixture caused a considerable decrease in the rate of inactivation by *p*-hydroxymercuribenzoate. For example, without Ca^{2+} ions all activity was lost in 5 min., but with Ca^{2+} ions only 20% of the activity was lost although apparently about 45% of the thiol groups had reacted.

Inhibitor studies with structural analogues and other compounds. Analogues of 3-hydroxybutyrate tested as possible competitive inhibitors of the enzyme are listed in Table 8. Their effect on the rate of oxidation of hydroxybutyrate at pH 6·5, 7·4 and 8·5 was measured. The K_i values for compounds showing appreciable inhibition were determined by the method of Dixon (1953) with two concentrations of 3-hydroxybutyrate. The results for the dicarboxylic acids (succinate and malonate) indicated a

Table 8. *Effect of various analogues on the rate of 3-hydroxybutyrate oxidation at different* pH *values*

The compounds listed below were tested in the following system: 100 μmoles of buffer of indicated pH, 3 μmoles of hydroxybutyrate, 1 μmole of NAD, 30 μmoles of compound to be tested and distilled water to a final volume of 3·0 ml. The reaction was started by the addition of enzyme (2·3 μg. of protein). The results are expressed as a percentage of the activity with hydroxybutyrate alone (100%).

Analogue	Activity (%) pH 6·5	pH 7·4	pH 8·5
Oxalate	100	100	100
Malonate	8	17	58
Succinate	29	70	100
Fumarate	100	100	100
Glutarate	79	92	100
DL-Malate	100	98	100
2-Hydroxyproprionate	100	100	80
DL-Lactate	29	13	12
L-Lactate	100	89	95
2-Hydroxybutyrate	50	29	35
2-Oxobutyrate	84	70	70
2-Oxopentanoate	100	97	100
2-Hydroxypentanoate	100	87	100
Laevulate	100	67	80
2-Aminobutyrate	100	100	100
Crotonate	100	100	100
Butyrate	100	100	100

complicated type of inhibition, but that by lactate, 2-hydroxybutyrate and laevulate was competitive (K_i values of 0·7 mM, 1·7 mM and 8·3 mM respectively at pH 7·4). It is assumed that the inhibition by DL-lactate is due to the D-isomer since L-lactate did not cause appreciable inhibition. As might be expected from their affinity for the enzyme, the 3-oxo homologues of acetoacetate act as competitive inhibitors of acetoacetate reduction; for example, at equimolar concentrations of acetoacetate and 3-oxo homologue (3·3 mM) there was an 85% inhibition with 3-oxopentanoate and a 50% inhibition with 3-oxohexanoate.

The metal-chelating agents, EDTA (10 mM) and *o*-phenanthroline (2·5 mM), did not inhibit when preincubated for 5 min. with the enzyme at pH 7·4.

DISCUSSION

It is of interest to compare the properties of the crystalline enzyme from *Rhodopseudomonas spheroides* with those of 3-hydroxybutyrate dehydrogenases from other sources and for this purpose a survey of the reported characteristics of the various enzyme preparations is given in Table 9. This survey also includes data on 3-hydroxy acid dehydrogenase (EC 1.1.1.45) (Smiley & Ashwell,

Table 9. *Survey of 3-hydroxybutyrate dehydrogenase from various sources*

For purposes of comparison, 3-hydroxy acid dehydrogenase from pig kidney is also included in the Table. References: [1]present paper; [2]Shuster & Doudoroff (1962); [3]Delafield, Cooksey & Doudoroff (1965); [4]Sekuzu *et al.* (1963); [5]Smiley & Ashwell (1961).

Source	*Rps. spheroides*[1]	*Rsp. rubrum*[2]	*Pseudomonas lemoignei*[3]	Ox heart[4]	Pig kidney[5]
Purity obtained	Crystalline (× 170)	× 180	× 120	× 80	× 100
Stability	Stable at 0°; dilute soln. unstable at 37°	Cold-labile	Stable at −20°	Stable at −20°	Depends on concentration
Optimum pH					
Oxidation reaction	8·0–9·2	6·8–8·5	8·0	8·0–8·5	8·5
Reduction reaction	6·0–6·8	6·2–6·8		7·0	6·3
Michaelis constants					
Acetoacetate	0·28 mM	0·071 mM	0·2 mM	—	6·4 mM
3-Hydroxybutyrate	0·41 mM	0·84 mM	0·6 mM	0·09 mM	5·3 mM
3-Oxopentanoate	0·031 mM	0·014 mM	—	—	—
NAD	0·08 mM	0·07 mM	—	0·11 mM	—
NADH	0·054 mM	—	—	—	—
Substrate specificity	D-3-Hydroxybutyrate	D-3-Hydroxybutyrate	D-3-Hydroxybutyrate	D-3-Hydroxybutyrate	L-3-Hydroxybutyrate and other L-3-hydroxy acids
	3-Hydroxypentanoate				
	Acetoacetate	Acetoacetate	Acetoacetate	Acetoacetate	Acetoacetate
	3-Oxopentanoate	3-Oxopentanoate	3-Oxopentanoate		
	3-Oxohexanoate	3-Oxohexanoate (?)	3-Oxohexanoate (?)		
Thiol inhibitors	Inhibition	No inhibition	No inhibition with fresh enzyme	Requires thiol for activity	Inhibition

1961), which can catalyse the reversible conversion of L(+)-3-hydroxybutyrate into acetoacetate; the mechanism of this reaction is presumably similar to that catalysed by 3-hydroxybutyrate dehydrogenase. Although insufficient information exists on the various preparations listed in Table 9, it is clear that, though they have properties in common, they differ widely in many respects.

Optimum pH. The pH optimum of the oxidation reaction (range: pH 8–9) and the reduction reaction (range: pH 6–7) are reasonably similar for all 3-hydroxybutyrate dehydrogenases and for 3-hydroxy acid dehydrogenase.

Michaelis constants. The K_m values of two of the bacterial enzymes for acetoacetate are of the same order, but that of the 3-hydroxy acid dehydrogenase is some 20-fold higher, which suggests that this enzyme does not play any major role in the metabolism of the oxo acid *in vivo*. There is a considerable difference between the affinity of the bacterial enzymes for 3-hydroxybutyrate and that of the enzyme from ox heart.

Substrate specificity. Only the enzymes from *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* have been examined in any detail for substrate specificity. Both enzymes react with 3-oxopentanoate and have a similar affinity for this substrate. Shuster & Doudoroff (1962) suggested, on the basis of kinetic studies, that the reaction of 3-hydroxybutyrate dehydrogenase with 3-oxohexanoate was due to contamination with acetoacetate. However, the fact that the enzyme described here reacts with both 3-hydroxy- and 3-oxo-hexanoate suggests that the C_6 homologues are true substrates for the dehydrogenase.

Inhibitor studies. The inhibition of the crystalline enzyme by compounds structurally related to hydroxybutyrate is of two types: (1) strictly competitive inhibition, which is most effective in the alkaline pH range, and (2) a mixed inhibition, which is more effective on decreasing the pH. The two most potent competitive inhibitors (DL-lactate and DL-2-hydroxybutyrate) contain a hydroxyl group in the 2-position and a terminal methyl group. Replacement of the hydroxyl group by hydrogen (as in butyrate) or by an amino group (as in 2-aminobutyrate) resulted in no inhibition. The effectiveness of the dicarboxylic acids as inhibitors decreased with increasing chain length. The same is true of the positional homologues of 3-hydroxybutyrate, since inhibition by 2-oxopentanoate and 2-hydroxypentanoate was not marked, and the affinity of the enzyme for 4-oxopentanoate (laevulate) was about 250-fold less than for 3-oxopentanoate. No comparable study has been carried out with the other 3-hydroxybutyrate dehydrogenases, except that Delafield *et al.* (1965) have reported inhibition of the enzyme from *Pseudomonas lemoignei* by DL-lactate and DL-2-hydroxybutyrate, and Berry (1964) has investigated the inhibition of the rat-liver enzyme by succinate.

The inhibition of the crystalline enzyme by low concentrations of thiol reagents suggests that thiol groups may be involved in the reaction catalysed by the dehydrogenase. The sensitive thiol groups react extremely rapidly as compared, for example, with the rate of combination of *p*-hydroxymercuribenzoate with the thiol groups thought to be involved in the active centre of lactate dehydrogenase (Dube, Roholt & Pressman, 1963). The protection of the enzyme by $NADH_2$ from the action of mercurials is a further indication that a thiol group(s) may be present in the active centre of this enzyme, because thiols include among their functions the binding of nucleotides to proteins. However, when the enzyme is protected by $NADH_2$ it appears that *p*-hydroxymercuribenzoate must bind with thiol groups outside the catalytic site because nearly twice as much mercurial is required to achieve a certain degree of inactivation (Table 7). Similar findings have been reported for lactate dehydrogenase by Di Sabato & Kaplan (1963).

Involvement of thiol groups has been demonstrated for both the mammalian 3-hydroxybutyrate dehydrogenase and 3-hydroxy acid dehydrogenase. In view of this it is surprising that no inhibition by thiol reagents has been found with preparations of the enzyme from other bacteria (Shuster & Doudoroff, 1962; Delafield *et al.* 1965) (Table 9). However, these authors do not state the conditions under which the preincubation with thiol reagent was carried out and it is possible that a component of the incubation mixture protected the enzyme from inactivation in their experiments.

Stability. The instability of dilute solutions of the crystalline enzyme at 37° is in contrast with its marked stability at 0°. Wise & Lehninger (1962) have presented evidence that the mitochondrial-bound 3-hydroxybutyrate dehydrogenase of rat liver is protected against inactivation at 30° by bound NAD (maintained in the reduced state with succinate), which in turn covers a thiol group(s) and protects this from oxidation. The results reported in this work on the protection of the crystalline enzyme by $NADH_2$ from heat inactivation and the action of thiol reagents appears to support this hypothesis. However, the marked protective action of Ca^{2+} ions suggests that in both instances protein conformation may be an important factor, since Ca^{2+} ions prevent the denaturation of a number of enzymes by maintaining their tertiary structure (Okunuki, 1961).

The enzyme from *Rhodospirillum rubrum* is cold-labile but that from *Pseudomonas lemoignei* is relatively stable in the cold. However, incubation of both enzymes with EDTA caused inactivation,

but the presence of Mg^{2+} ions or NAD protected the enzymes as did high protein concentration. These results, together with those reported for the crystalline enzyme, suggest that the instability of the bacterial enzymes under various conditions may be the result of their dissociation to constituent sub-units, and that this can be prevented by the presence of bivalent cations or by the binding of nicotinamide–adenine dinucleotides.

We thank Mr C. J. Teal for carrying out the molecular-weight measurements.

REFERENCES

Adickes, F. & Andresen, G. (1944). *Liebigs Ann.* **555**, 41.
Beisenherz, G., Boltze, H. J., Bücher, Th., Czok, R., Garbade, K. H., Meyer-Arendt, E. & Pfleiderer, G. (1953). *Z. Naturf.* **8b**, 555.
Bergmeyer, H.-U. & Bernt, E. (1965). *Enzymol. biol. & clin.* **5**, 65.
Bergmeyer, H.-U., Holz, G., Kauder, E. M., Möllering, H. & Wieland, O. (1961). *Biochem. Z.* **333**, 471.
Berry, M. N. (1964). *Biochim. biophys. Acta*, **92**, 156.
Boyer, P. D. (1954). *J. Amer. chem. Soc.* **76**, 4331.
Delafield, F. P., Cooksey, K. E. & Doudoroff, M. (1965). *J. biol. Chem.* **240**, 4023.
Di Sabato, G. & Kaplan, N. O. (1963). *Biochemistry*, **2**, 776.
Dixon, M. (1953). *Biochem. J.* **55**, 170.
Dube, S. K., Roholt, O. & Pressman, D. (1963). *J. biol. Chem.* **238**, 613.
Edson, N. L. (1935). *Biochem. J.* **29**, 2082.
Hall, L. M. (1962). *Analyt. Biochem.* **3**, 75.
Krebs, H. A. (1961). *Biochem. Prep.* **8**, 75.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Okunuki, K. (1961). *Advanc. Enzymol.* **23**, 29.
Sekuzu, I., Jurtshuk, P. & Green, D. E. (1963). *J. biol. Chem.* **238**, 975.
Shuster, C. W. & Doudoroff, M. (1962). *J. biol. Chem.* **237**, 603.
Smiley, J. D. & Ashwell, G. (1961). *J. biol. Chem.* **236**, 357.
Williamson, D. H., Mellanby, J. H. & Krebs, H. A. (1962). *Biochem. J.* **82**, 90.
Wise, J. B. & Lehninger, A. L. (1962). *J. biol. Chem.* **237**, 1363.
Yphantis, D. A. (1960). *Ann. N.Y. Acad. Sci.* **88**, 586.