gradient on the addition of potassium to the suspension medium as reported for three strains examined previously.

2. When cells were alternately exposed to water and potassium chloride solutions they lost and gained potassium repeatedly though the amounts taken up decreased slightly on repeated washing. Thus intracellular soluble cations do not seem to play a major part in the binding of potassium by exchanging with the potassium of the medium.

3. Cells suspended in hypo-osmotic solutions swelled to about twice the original volume but the loss of potassium was independent of swelling and hypo-osmoticity; it also occurred in sucrose solution iso-osmolar with the growth medium. The aerobic uptake of potassium was approximately the same from hypo-osmotic media and from iso-osmolar sucrose solutions.

4. Octan-2-ol prevented the active transport of potassium but had no major effect on the anaerobic uptake.

5. Electrotitration of the medium showed that small quantities of nitrogenous bases were discharged when potassium was taken up. These were equivalent to less than 10% of the potassium uptake.

6. The uptake of potassium from potassium chloride solutions was accompanied by a discharge of H^+ ions, causing the pH of the medium to fall by about 0·5 unit. The release of H^+ ions was equivalent to no more than a small fraction of the potassium uptake.

7. When cells were suspended in dilute potassium phosphate buffer the uptake of potassium was accompanied by a fall of pH of 0·46 unit and an uptake of phosphate against a gradient. Calculations show that in this case electroneutrality was partly maintained by an exchange of K^+ for H^+ ions, partly by the movement of phosphate, each factor contributing about 50%. Analogous results were obtained when potassium bicarbonate was added and the bicarbonate form of a cation-exchange resin was used as a buffer.

8. Chloroform–methanol-extractable cell lipids did not bind significant amounts of potassium.

9. Cells disrupted by the Hughes press or ultrasonically when placed in a dialysing sac were still able to bind potassium. It is concluded that a colloidal cell constituent rather than specific structures effects the binding.

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Enzymic Determination of D(−)-β-Hydroxybutyric Acid and Acetoacetic Acid in Blood

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The current methods for the estimation of β-hydroxybutyrate (e.g. Greenberg & Lester, 1944; Thin & Robertson, 1952; Bloom, 1958; Bessman & Anderson, 1957) all involve its oxidation to acetone and the estimation of the latter. These methods are laborious and have several unsatisfactory features. They are unspecific in that other compounds also yield acetone on oxidation and they do not differentiate between the optical isomers. Moreover, the conversion of β-hydroxybutyrate into acetone is not quantitative and the yield varies with the conditions. The method described in this paper is in principle more specific and more rapid than the older procedures. It is analogous to the enzymic determination of many other metabolites. It depends on the spectrophotometric measurement of

the reduction of diphosphopyridine nucleotide by D(−)-β-hydroxybutyrate in the presence of D(−)-β-hydroxybutyric dehydrogenase.

The D(−)-β-hydroxybutyric dehydrogenase of mammalian tissues which was characterized by Green, Dewan & Leloir (1937) is unsuitable as an analytical tool because it is insoluble and relatively unstable. A soluble D(−)-β-hydroxybutyric dehydrogenase has recently been discovered in certain micro-organisms which accumulate the polymer of β-hydroxybutyrate (Gavard, Combre & Tuffet, 1960; Doudoroff, Merrick & Contopoulou, 1961; Carr & Lascelles, 1961). A partially purified preparation of this enzyme from *Rhodopseudomonas spheroides* has proved satisfactory for analytical purposes. The enzyme catalyses the reversible reaction:

$$\text{D}(-)\text{-}\beta\text{-hydroxybutyrate} + \text{DPN}^+ \rightleftharpoons \text{acetoacetate} + \text{DPNH} + \text{H}^+. \quad (1)$$

The equilibrium constant of this reaction is $1{\cdot}42 \times 10^{-9}$ at 25° (Krebs, Mellanby & Williamson, 1962). Thus at pH 8·5 the reaction goes readily to completion from left to right if the acetoacetate is removed by a ketone-trapping agent, such as hydrazine. On the other hand, in the presence of excess of reduced diphosphopyridine nucleotide at pH 7, the reduction of acetoacetate is virtually quantitative. The enzyme may therefore be used to determine both D(−)-β-hydroxybutyrate and acetoacetate.

EXPERIMENTAL AND RESULTS

Acetoacetate was prepared by hydrolysis of freshly distilled ethyl acetoacetate according to the method of Ljunggren (1924). After neutralization and removal of ethanol the resulting solution of acetoacetate was standardized manometrically by the method of Edson (1935).

Oxaloacetate was prepared by the methods of Wohl & Oesterlin (1901) and Wohl & Claussner (1907). It was dissolved in water and neutralized with NaOH immediately before use. Sodium DL-β-hydroxybutyrate was obtained from British Drug Houses Ltd. The D(−)-β-hydroxybutyrate was a gift from Dr Greville (Lehninger & Greville, 1953). Malic dehydrogenase, DPN^+ and DPNH were obtained from C. F. Boehringer und Soehne GmbH., Mannheim, Germany.

Culture of Rhodopseudomonas spheroides. The strain of *R. spheroides* originated from the National Collection of Industrial Bacteria (N.C.I.B. no. 8253). The organism was grown aerobically in the dark at 30°, in a culture medium adapted from that of Lascelles (1956). One litre contained: KH_2PO_4, 5 g.; K_2HPO_4, 5 g.; $MgSO_4,7H_2O$, 0·2 g.; $(NH_4)_2HPO_4$, 0·8 g.; sodium glutamate, 2 g.; sodium acetate trihydrate, 3 g.; $CaCl_2$, 40 mg.; ferric citrate, 30 mg.; nicotinic acid, 1 mg.; thiamine, 1 mg.; $MnSO_4,4H_2O$, 1 mg.; biotin, 100 μg. The pH was 6·9. Batches of cells were grown for 48 hr. in 10-l. aspirators containing 8 l. of medium. The culture was aerated at a rate of approximately half a litre per minute through a sintered-glass distributer tube (porosity 2). Each aspirator was inoculated with 250 ml. of a 24 hr. culture, grown in the basal medium supplemented with yeast extract, under the conditions described by Lascelles (1959). The cells were pink, owing to the formation of the photopigments characteristic of this organism. They were collected on a Sharples centrifuge. The cell paste from each aspirator was washed twice with a total volume of 2 l. of 10 mM-potassium phosphate buffer, pH 7·6, and suspended in 150 ml. of this buffer. The average yield of cells grown for 48 hr. under the above conditions was 7 g. dry wt. per aspirator. The cells retained most of their activity after storage in the deep-freeze for at least 6 months.

Larger quantities of cells (100 l. cultures) were grown by Mr Elsworth at the Microbiological Research Establishment, Porton, Wilts.

Preparation of cell-free extracts. Cell suspension (300 ml.; from two aspirators), contained in a stout glass jar packed in ice, were exposed to ultrasonic vibration at 19–20 kcyc./sec. for 15 min. in an ultrasonic generator (Measuring and Scientific Equipment, Spenser Street, London, S.W. 1). The sonically disrupted cells were centrifuged at 15000*g* for 10 min. at 0°, to remove cell debris as well as the polymer of β-hydroxybutyrate which accumulates in *R. spheroides* grown under these conditions. The red supernatant fluid was used for the preparation of the enzyme.

Assay of D(−)-β-*hydroxybutyric dehydrogenase.* The spectrophotometric assay is based on the decrease in extinction at 340 mμ which takes place when DPNH is oxidized by acetoacetate in the presence of the dehydrogenase. The procedure was as follows: a spectrophotometer cell of 1 cm. light-path contained: tris buffer, 100 μmoles (1 ml.; 0·1 M), pH 7·6; DPNH, 0·5 μmole (0·1 ml.; 5 mM); water to a final volume of 3 ml. The temperature was 20–25°. At zero time, 0·1 ml. or less of the enzyme solution was added. After thorough mixing the extinction was read and 10 μmoles (0·05 ml.; 0·2 M) of acetoacetate were added. The contents of the cell were mixed again and readings were taken at 1 min. intervals. One unit of enzyme activity is defined as that which causes a decrease in extinction at 340 mμ of 0·01/min. under the test conditions. Protein was determined by the biuret method (Gornall, Bardawill & David, 1949). For each preparation there was good proportionality between the amount of protein added and the

activity found (between 1 and 10 units). The specific activity of the dehydrogenase is expressed as units of enzyme activity/mg. of protein.

When acetoacetate was omitted from the assay system, the initial crude extracts slowly oxidized DPNH. A correction for this oxidation was applied.

Preparation of the D(−)-*β-hydroxybutyric dehydrogenase.* The routine preparation of the dehydrogenase was carried out at 2–4°, except where otherwise stated, and involved three stages:

Stage I: ammonium sulphate fractionation. To every 100 ml. of the cell-free supernatant, 66 ml. of $(NH_4)_2SO_4$ saturated at 0°, pH 7·7, containing mM-EDTA was added at 2° over a period of 15 min. The precipitate was removed by centrifuging at 10000*g* for 10 min. To the supernatant derived from 100 ml. of the original extract, a further 120 ml. of the $(NH_4)_2SO_4$ was added, and the mixture was left for 1 hr. The precipitate was collected by centrifuging for 10 min. at 10000*g*.

Stage II: ethanol fractionation. The precipitate from stage I was dissolved in 50 mM-potassium phosphate buffer, pH 7·4, containing 10 mM-magnesium acetate, and the protein concentration was adjusted to between 4 and 5 mg./ml. The precipitate that appeared on dilution was removed by centrifuging. The supernatant, contained in a beaker, was cooled to −2° in a solid CO_2–acetone bath. While the mixture was stirred continuously with a magnetic stirrer, ethanol, cooled to −15°, was introduced beneath the surface over a period of 20 min., in the proportion of 43 ml. to 100 ml. of supernatant. The small amount of precipitated protein was removed by centrifuging for 15 min. at 15000*g*.

Stage III: ammonium sulphate fractionation. To every 100 ml. of the supernatant from stage II, 186 ml. of saturated $(NH_4)_2SO_4$ solution was added. The precipitate was centrifuged off and discarded. A further 114 ml. of $(NH_4)_2SO_4$ solution was added to the resulting supernatant and the mixture was left overnight. The precipitate was collected by centrifuging at 15000*g* for 20 min. and dissolved in 5 ml. of 0·01 M-potassium phosphate buffer, pH 7·4, for every 100 ml. of original cell-free extract. The D(−)-*β*-hydroxybutyric-dehydrogenase activity of this solution was determined, and the volume adjusted with the same buffer to give 1000 units of activity/ml. Such solutions were used in the estimation of D(−)-*β*-hydroxybutyrate and acetoacetate and retained most of their activity after storage at 2–4° for at least 1 month.

At no stage in the purification procedure did the addition of $MnCl_2$ or $MgCl_2$ (0·1–1 mM) increase the activity of the D(−)-*β*-hydroxybutyric dehydrogenase. Even after prolonged dialysis of the final enzyme preparation against 10 mM-potassium phosphate buffer, pH 7·4, no requirement for these bivalent cations could be demonstrated. The D(−)-*β*-hydroxybutyric-dehydrogenase activity in the final preparation was unaffected by EDTA (0·1–1 mM). In these respects this D(−)-*β*-hydroxybutyric dehydrogenase differed from that obtained from *Rhodospirillum rubrum* by Doudoroff *et al.* (1961) and that from *Bacillus megaterium* (Gavard *et al.* 1960).

Purity of the D(−)-*β-hydroxybutyric-dehydrogenase preparation.* The specific activity of the dehydrogenase in the final solution was usually ten times that in the initial cell-free extract (Table 1). The initial supernatant contained several pyridine nucleotide-linked dehydrogenases (lactic, glutamic, malic and polyol dehydrogenases) and DPNH oxidase, which in the presence of their respective substrates could interfere with the estimation of D(−)-*β*-hydroxybutyrate and acetoacetate. The purification procedure removes lactic and glutamic dehydrogenases and DPNH oxidase, but occasionally leaves traces of polyol (mannitol, sorbitol) dehydrogenase and a variable amount of the malic dehydrogenase. The physical characteristics of the latter are similar to those of the D(−)-*β*-hydroxybutyric dehydrogenase, and it has not yet been possible to separate them completely (Table 2). In the initial extract, the malic-dehydrogenase activity was 10–30 times that of the D(−)-*β*-hydroxybutyric dehydrogenase, whereas the final preparation contained less than 10% of the initial malic-dehydrogenase activity. Thus the ratio of activity of this enzyme to that of D(−)-*β*-hydroxybutyric dehydrogenase was decreased to between 1 and 3.

Estimation of D(−)-*β-hydroxybutyrate.* Among various ketone-trapping agents tested hydrazine was found to be the best to drive reaction (1) from left to right. To a spectrophotometer cell of

Table 1. *Purification of* D(−)-*β-hydroxybutyric dehydrogenase*

For description of fractions and definition of units see text.

Fraction	Volume (ml.)	Total units	Specific activity (units/mg. of protein)	Recovery (%)
Cell-free extract	250	60 000	15	—
Solution of precipitate from stage I	240	44 000	43	73
Final solution from stage III	12·5	20 000	142	30

1 cm. light-path are added: tris buffer, 50 μmoles (0·5 ml.; 0·1 M), pH 8·5; hydrazine hydrate, 1 m-mole, pH 8·5 (1 ml. of the following: 1 ml. hydrazine hydrate, 5 ml. of N-HCl, diluted to 20 ml. with water); DPN^+, 1·0 μmole (0·1 ml.; 10 mM); D(−)-β-hydroxybutyrate sample, containing 0·02–0·2 μmole; water to a final volume of 3 ml. A control cell contains all reagents except the unknown sample. The extinction is read at 340 mμ against the control at 2 min. intervals until constant readings are obtained. An enzyme preparation (0·025 ml.) containing about 1000 units of activity/ml. is then added, the solutions are mixed and readings are taken at 10 min. intervals until the extinction is again constant. The reaction takes 40–60 min. to reach completion.

The amount of D(−)-β-hydroxybutyrate present in the sample is calculated from the increase in extinction, the extinction coefficient of DPNH at 340 mμ being taken as 6·22 $cm.^2/\mu mole$ (Horecker & Kornberg, 1948). With pure solutions of D(−)-β-hydroxybutyrate, the observed increases in extinction were within ± 5 % of the calculated theoretical values. In each series of estimations, a D(−)-β-hydroxybutyrate standard, containing 0·2 μmole, is included. If sufficient spectrophotometer cells are available, 12 or more determinations may be carried out simultaneously.

Because of the contamination of the purified D(−)-β-hydroxybutyric dehydrogenase with DPN-linked malic and polyol dehydrogenases (Table 2), the substrates of these enzymes may be expected to interfere with the estimation of D(−)-β-hydroxybutyrate in two ways: L(−)-malate, sorbitol and mannitol can reduce DPN^+ and cause the value for β-hydroxybutyrate to be too high, or oxaloacetate and fructose can reoxidize the DPNH formed and give results that are too low. In order to assess the extent of possible interference, a standard amount of D(−)-β-hydroxybutyrate was determined in the presence of various substrates (Table 3). The values obtained were 7 % too high with L-malate when its concentration was equal to that of D(−)-β-hydroxybutyrate. The following substances (final concentration 3 mM) when tested in the usual system, but in the absence of hydrazine, caused no reduction of DPN^+ and did not inhibit its reduction by D(−)-β-hydroxybutyrate: D-tartrate; tartronate; DL-glycerate; mevalonate; *n*-butyrate; *n*-valerate; *n*-hexanoate; *n*-octanoate; palmitate; stearate; arachidate; crotonate; acetate; α- and β-glycerophosphate; DL-serine; hypoxanthine; DL-threonine. A mixture of amino acids in the form of a casein digest (final concentration 0·05 %) did not react with or inhibit the enzyme. The following substances (final concentration 3 mM) did not reduce DPN^+ but gave approximately a 50 % inhibition of D(−)-β-hydroxybutyric dehydrogenase when present in the final concentration indicated: *n*-decanoate, 1 mM; *n*-undecylenate, 0·06 mM; laurate, 3 mM; oleate, 3 mM; linoleate, 0·15 mM.

Preliminary experiments were carried out with three homologues of β-hydroxybutyrate. β-Hydroxypropionate did not react with or inhibit β-hydroxybutyric dehydrogenase. β-Hydroxyhexanoate and β-hydroxyvalerate reduced DPN^+ at about one-tenth the rate at which β-hydroxybutyrate reduced it, but, when added to β-hydroxybutyrate at the same concentration, β-hydroxyvalerate

Table 2. *Enzymic impurities in D(−)-β-hydroxybutyric-dehydrogenase preparation at stage III of purification*

The enzymes were assayed as described under 'Assay of D(−)-β-hydroxybutyric dehydrogenase' by replacing acetoacetate with the appropriate substrate. The units are defined as for D(−)-β-hydroxybutyric dehydrogenase.

Enzyme	Substrate	Units/ml.
D(−)-β-Hydroxybutyric dehydrogenase	Acetoacetate	2040
Malic dehydrogenase	Oxaloacetate	3000
Glutamic dehydrogenase	α-Oxoglutarate	10
Polyol dehydrogenase	D-Fructose	52
Lactic dehydrogenase	Pyruvate	0
DPNH oxidase	DPNH	0

Table 3. *Interference with the determination of D(−)-β-hydroxybutyrate*

D(−)-β-Hydroxybutyrate (0·2 μmole) was determined under the standard conditions in the presence of the indicated substrates. No interference was found from lactate, pyruvate or α-oxoglutarate.

Substrate added	Amount (μmole)	Reading after 60 min. $E_{340\,m\mu}$	Reading after 60 min. Error due to second substrate (%)	Reading after 100 min. $E_{340\,m\mu}$	Reading after 100 min. Error due to second substrate (%)
None	—	0·410	—	0·415	—
D-Fructose	1·0	0·386	−6	0·386	−7
Sorbitol	1·0	0·445	+9	0·465	+12
L-Malate	0·2	0·442	+7·5	0·462	+11
L-Malate	1·0	0·531	+29	0·571	+38

decreased the rate of reduction of DPN^+ to half that with β-hydroxybutyrate alone.

Estimation of acetoacetate. To a spectrophotometer cell of 1 cm. light-path are added 100 μmoles (1 ml.; 0·1 M) of potassium phosphate buffer, pH 7·0, 0·5 μmole (0·1 ml.; 5 mM) of DPNH, acetoacetate sample, containing 0·02–0·2 μmole and water to a final volume of 3 ml. A control cell contains all reagents except the unknown sample. The extinction at 340 mμ is read against a water blank. The D(−)-β-hydroxybutyric-dehydrogenase preparation (0·025 ml.) is added, and readings are taken at 5 min. intervals until no further decrease in extinction occurs. The reaction is completed within 20–30 min. The calculation is analogous to that for the estimation of D(−)-β-hydroxybutyrate. A correction is applied for any absorption changes in the control cell. With pure solutions of acetoacetate, the observed decreases in extinction were within ±5% of the calculated values. In each series of estimations, a standard containing 0·2 μmole of acetoacetate is included. Owing to the presence of malic and polyol dehydrogenases in the enzyme preparation, oxaloacetate and fructose might be expected to oxidize DPNH and hence give values for acetoacetate which are too high. Oxaloacetate can be removed by preliminary incubation with malic dehydrogenase and DPNH. Owing to the low concentrations of oxaloacetate in blood and its instability, this is not usually necessary.

If acetoacetate is determined in the presence of relatively high concentrations (25–50-fold) of D(−)-β-hydroxybutyrate the values obtained are too low, owing to the displacement of the equilibrium of the system (Table 4). In blood, the ratio of D(−)-β-hydroxybutyrate to acetoacetate is usually below four (see Table 5).

Application of the methods to blood

Preliminary treatment. Heparin does not interfere with either of the determinations and was always used as an anticoagulant. Blood samples must be cooled to 2–4° soon after collection to minimize loss of acetoacetate. The loss is illustrated by the following experiments. When human blood was kept in air at 20°, 10% of its acetoacetate disappeared within 1 hr.; after 18 hr. only 55% was left, whereas at 0° there was no detectable loss after 18 hr. When dog, human or guinea-pig blood to which had been added 1 μmole of acetoacetate/ml. was incubated at 37° in air containing 5% of carbon dioxide, about 40% of the acetoacetate disappeared within 1 hr. and no D(−)-β-hydroxybutyrate accumulated. The oxygen uptake of blood is insufficient to account for the oxidation of the acetoacetate removed and it was therefore probably non-enzymically decarboxylated (Rossi, 1938).

Deproteinization. Ice-cold 30% (w/v) perchloric acid (3 ml.) was added to 3 ml. of blood in a centrifuge tube. After thorough mixing, the precipitated protein was removed by centrifuging. A measured volume of the supernatant was neutralized with 20% (w/v) potassium hydroxide and the volume of potassium hydroxide used noted. The mixture was kept at 2° for 30 min. and the insoluble potassium perchlorate centrifuged off. The determination of D(−)-β-hydroxybutyrate and acetoacetate was carried out on the resulting supernatant.

Recovery of D(−)-β-hydroxybutyrate and acetoacetate added to blood. The initial concentrations of D(−)-β-hydroxybutyrate and acetoacetate were determined in a sample of blood. Known amounts (0·17–1·0 μmole/ml. of blood) of D(−)-β-hydroxybutyrate and acetoacetate were added to further portions of this blood. The blood was deproteinized by the standard procedure, and the D(−)-β-hydroxybutyrate and acetoacetate concentrations were determined. After correcting for the initial concentrations, the percentage recovery of D(−)-β-hydroxybutyrate was 104 ± 5·75 (S.D.) and of acetoacetate was 99 ± 6·25 (S.D.).

Concentration of D(−)-β-hydroxybutyrate and acetoacetate in fasting human subjects. Forty-nine samples from 16 human subjects who had fasted for 12–24 hr. and who had no obvious metabolic

Table 4. *Interference with the determination of acetoacetate*

Acetoacetate (0·18 μmole) was determined under the standard conditions in the presence of the indicated substrates. No interference was found from pyruvate or α-oxoglutarate.

		Reading after 10 min.		Reading after 30 min.	
Substrate added	Amount (μmoles)	$E_{340\,m\mu}$	Error due to second substrate (%)	$E_{340\,m\mu}$	Error due to second substrate (%)
None	—	0·357	—	0·362	—
Oxaloacetate	0·2	0·800	+124	0·805	+122
D-Fructose	1·0	0·360	+ 1	0·388	+ 5
D(−)-β-Hydroxybutyrate	1·0	0·335	− 6	0·351	− 3
D(−)-β-Hydroxybutyrate	5·0	0·310	− 13	0·320	− 12·5
D(−)-β-Hydroxybutyrate	10·0	0·260	− 27	0·267	− 26

Table 5. *D(−)-β-Hydroxybutyrate and acetoacetate in the blood of human subjects who had fasted from 12 to 20 hr.*

The values given are averages and the number of samples refers to the estimations carried out on the same patient on different days. The blood was treated as described in the text. ECT refers to patients about to undergo electro-convulsive therapy.

Age (years)	Sex	Clinical condition	No. of samples	Aceto-acetate (μmole/ml.)	D(−)-β-Hydroxy-butyrate (μmole/ml.)	Sum of ketone bodies (μmole/ml.)	β-Hydroxybutyrate / Acetoacetate
42	F	Depression (ECT)	5	0·015	0·031	0·046	1·78
34	M	Chronic schizophrenia (ECT)	3	0·046	0·065	0·111	1·40
50	F	Cervical polyp	1	0·038	0·100	0·138	2·6
49	F	Depression (ECT)	3	0·049	0·103	0·152	2·10
48	F	Uterine prolapse	1	0·035	0·151	0·186	4·3
57	M	Depression (ECT)	6	0·063	0·144	0·207±0·102 (S.D.)	2·28±0·57 (S.D.)
47	F	Depression (ECT)	3	0·064	0·169	0·233	2·64
61	F	Depression. Ménière's disease (ECT)	7	0·069	0·200	0·269±0·076 (S.D.)	2·9±0·61 (S.D.)
48	F	Depression (ECT)	1	0·063	0·211	0·274	3·36
23	F	Depression (ECT)	3	0·066	0·211	0·277	3·2
67	M	Depression (ECT)	5	0·118	0·224	0·342±0·129 (S.D.)	1·9±0·31 (S.D.)
45	M	Depression (ECT)	6	0·115	0·365	0·480±0·139 (S.D.)	3·2±0·65 (S.D.)
68	M	Carcinoma of bladder	1	0·142	0·376	0·518	2·65
68	M	Depression (ECT)	2	0·138	0·438	0·576	3·17
69	M	Inguinal hernia	1	0·175	0·587	0·762	3·35
63	M	Ulcerative colitis	1	0·226	0·650	0·876	2·87

abnormalities were analysed for β-hydroxybutyrate and acetoacetate. The subjects were all patients of the United Oxford Hospitals. Some were about to undergo surgical operations and others electro-convulsive therapy. Details of the clinical condition and the results of the determinations are shown in Table 5. The total ketone-body concentration varied between 0·046 and 0·876 μmole/ml. and the ratio of β-hydroxybutyrate to acetoacetate ranged from 1·4 to 4·3, the average being 2·73 ± 0·73 (S.D.). The ratio was independent of the total ketone-body concentration and there was relatively little variation in the ratio in different samples from the same individual taken on different days. The average value of the ratio is a little higher than that obtained by Friedemann (1942), who found an average of 2·01 on five human subjects.

DISCUSSION

The main advantages of the enzymic method are its greater specificity and convenience. As twelve determinations can be carried out within 1 hr., the time taken is rather less than one-tenth of the time required for the procedure of Greenberg & Lester (1944). At present the main shortcoming of the method is the contamination of the purified enzyme with malic dehydrogenase. Although 90–95% of the contaminating enzyme is removed by the purification procedure, the remaining traces have so far proved inseparable from β-hydroxybutyric dehydrogenase. This contamination is not of major importance as long as the amount of malate present does not exceed that of β-hydroxybutyrate by a factor of more than 2 because under the test conditions the reduction of DPN^+ by malate is much slower than the reduction by β-hydroxybutyrate. It is therefore possible to correct by extrapolation for the slow reduction of DPN^+ at low malate concentrations. The concentration of malate in blood is of the order of 0·03 μmole/ml. (Hummel, 1949) and this is within the range where it does not interfere. The application of the methods to extracts of other animal tissues requires further investigation.

The acetoacetate values obtained by the enzymic method are in agreement with those obtained by the colorimetric method of Walker (1954). The enzymic method is more rapid with approximately the same range of sensitivity.

SUMMARY

1. D(−)-β-Hydroxybutyric dehydrogenase of *Rhodopseudomonas spheroides* has been partially purified. The enzyme is water-soluble and stable. The purified material is free from reduced diphosphopyridine nucleotide oxidase but still contains

some malic and polyol (sorbitol, mannitol) dehydrogenase.

2. Unlike the similar enzymes from *Rhodospirillum rubrum* and *Bacillus megaterium*, the activity of the purified enzyme is not increased by the addition of magnesium or manganese.

3. The use of the enzyme for the spectrophotometric determination of D(−)-β-hydroxybutyrate in 0·02–0·20 μmole quantities is described. The reduction of diphosphopyridine nucleotide is measured at pH 8·5 in the presence of hydrazine.

4. The enzyme can also be used for the estimation of acetoacetate by measuring the disappearance of reduced diphosphopyridine nucleotide at pH 7·0.

5. The ratio of D(−)-β-hydroxybutyrate to acetoacetate in fasting human subjects was found to be 2·73 ± 0·73 (S.D.) irrespective of total concentration of ketone bodies over the range 0·046–0·876 μmole/ml.

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The Equilibrium Constant of the β-Hydroxybutyric-Dehydrogenase System

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In view of the importance of the ketone bodies in intermediary metabolism, information on the redox potential of the β-hydroxybutyrate–acetoacetate system is of general interest. Green, Dewan & Leloir (1937) measured the potential of this system electrometrically in the presence of β-hydroxybutyric dehydrogenase and a mediator and obtained a value for E'_0 of −0·282 v at pH 7·0 and 38°. Hoff-Jörgensen (1938), using similar techniques and the same conditions, arrived at a value of −0·293 v. Green & Dewan (1937) reported data on the equilibrium concentrations of β-hydroxybutyrate, acetoacetate and the reduced and oxidized forms of diphosphopyridine nucleotide. These data indicate an equilibrium constant:

$$K = \frac{[\text{acetoacetate}]\,[\text{DPNH}]\,[\text{H}^+]}{[\beta\text{-hydroxybutyrate}]\,[\text{DPN}^+]}$$

of between $1{\cdot}1 \times 10^{-7}$ and $4{\cdot}3 \times 10^{-7}$. From these figures and the E'_0 value for the diphosphopyridine nucleotide system established by Burton & Wilson (1953), Burton (1957) calculated the E'_0 value for the β-hydroxybutyrate–acetoacetate system to be −0·349 v (pH 7·0; 25°). The enzyme used in these