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 μ mole 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 μ mole/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-Jørgensen (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 -hydroxy-

butyrate, 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 1.1×10^{-7} and 4.3×10^{-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

Table 1. Values for the equilibrium constant of the D(−)-β-hydroxybutyric-dehydrogenase system

To a spectrophotometer cell were added: 100 μmoles of tris buffer; a known amount of DPN⁺ or DPNH (about 0.5 μmole); a known amount of DL-β-hydroxybutyrate or acetoacetate (0.2–1.0 μmole); water to a final volume of 3.3 ml. The extinction was read at 340 mμ and 0.025 ml. of enzyme solution (250 μg. of protein) was added. After mixing, readings were taken at 5 min. intervals until equilibrium was reached. Temperature, 25°. In series *A* the initial substrates were acetoacetate and DPNH, in series *B* β-hydroxybutyrate and DPN⁺.

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

		Concentration at equilibrium						Series
Buffer	pH	H ⁺ (μmM)	Aceto- acetate (μM)	DPNH (μM)	D(−)-β- Hydroxy- butyrate (μM)	DPN ⁺ (μM)	K (μmM)	
Tris	8.70	1.99	54.0	54.0	36.0	133	1.21	<i>B</i>
Tris	8.55	2.82	38.1	38.1	24.0	145	1.18	<i>B</i>
Tris	8.10	7.94	29.2	29.2	32.8	154	1.34	<i>B</i>
Tris	8.10	7.94	15.0	72.0	66.9	74.4	1.74	<i>A</i>
Tris	7.70	19.9	35.7	35.7	119	148	1.45	<i>B</i>
Tris	7.70	19.9	3.30	65.9	52.0	53.5	1.55	<i>A</i>
Tris	7.70	19.9	3.45	66.9	52.3	54.6	1.61	<i>A</i>
Tris	7.60	25.1	23.7	23.7	66.3	163	1.29	<i>B</i>
Tris	7.40	39.8	4.80	49.2	77.1	84.3	1.45	<i>A</i>
Tris	7.40	39.8	33.0	33.0	258	154	1.09	<i>B</i>
Tris	7.40	39.8	37.5	37.5	273	145	1.41	<i>B</i>
Tris	7.40	39.8	4.60	48.1	76.6	83.2	1.38	<i>A</i>
Phosphate	7.05	89.0	29.0	29.0	281	154	1.72	<i>B</i>
Phosphate	7.00	100	25.5	25.5	274	162	1.46	<i>B</i>

Mean of *K*: 1.42 ± 0.19 (S.D.)

measurements was the β-hydroxybutyric dehydrogenase of animal tissues and accurate measurements in its presence are difficult owing to its insolubility and instability. The β-hydroxybutyric dehydrogenase of bacterial origin, recently discovered in certain micro-organisms that accumulate poly-β-hydroxybutyrate ester, provides a better tool for the accurate measurement of the equilibrium constant because it is soluble, very stable and can be freed from DPNH oxidase. Data reported in this paper show that *K* calculated from the data by Green & Dewan (1937) is incorrect by a factor of about 100. The electrometrically obtained data of the previous investigators are nearer the true value of the redox potential.

MATERIALS AND METHODS

Acetoacetate was prepared from its ethyl ester according to Ljunggren (1924). After neutralization and removal of ethanol the solution was standardized manometrically by the method of Edson (1935). Sodium DL-β-hydroxybutyrate was obtained from British Drug Houses Ltd. It was assayed enzymically and on the assumption that the L-component did not react and that the salt contained 1 molecule of water of crystallization the purity was at least 98–99 %. The bacterial D(−)-β-hydroxybutyric dehydrogenase was prepared from *Rhodospseudomonas spheroides* as described by

Williamson, Mellanby & Krebs (1962). An enzyme preparation purified by ammonium sulphate and ethanol fractionation was used.

DPN⁺ and DPNH were obtained from C. F. Boehringer und Soehne GmbH., Mannheim, Germany. Their concentrations in solutions were assayed with alcohol dehydrogenase according to the method of Colowick, Kaplan & Ciotti (1951). The extinction coefficient of DPNH was taken to be 6.22 cm.²/μmole.

Spectrophotometric measurements were made in the Unicam SP. 500 instrument fitted with a constant-temperature cell.

Measurement of the equilibrium constant. The equilibrium of the reaction:

was approached from either side at different pH values. The results are shown in Table 1. In series *A* the enzyme was added to a solution containing known amounts of acetoacetate and DPNH dissolved in buffer solutions. The disappearance of DPNH was measured at 340 mμ until its concentration remained constant. From the amounts of DPNH removed and the known initial concentrations of acetoacetate and DPNH, the concentrations of DPN⁺ and D(−)-β-hydroxybutyrate were calculated. In series *B* the enzyme was added to solutions containing known amounts of DL-β-

hydroxybutyrate and DPN^+ and the amounts of DPNH formed were measured. Phosphate buffer was used at pH 7 and tris buffer at the more alkaline pH values. Controls containing all reagents except β -hydroxybutyrate or acetoacetate showed no significant changes in extinction.

The results of 14 individual measurements showed good agreement (Table 1). The mean value for K was 1.42 ± 0.19 (S.D.) $\times 10^{-9}$. From this value and the E'_0 for the DPN^+ -DPNH system of -0.320 v at pH 7.0 and 25° (Burton, 1957), E'_0 for the β -hydroxybutyrate system is calculated to be -0.266 v. This figure is much less negative than that calculated by Burton (1957) and slightly less negative than those arrived at (for 38°) by Green *et al.* and Hoff-Jørgensen. For a full discussion of any error in Burton's figure for E'_0 of the DPN system see Clark (1960); the figure of -0.320 v may be a little too high. The above K value is very similar to that recently obtained at 32° by Doudoroff, Merrick & Contopoulou (1961), using a preparation of β -hydroxybutyric dehydrogenase from *Rhodospirillum rubrum* (1.3×10^{-9}).

SUMMARY

1. The equilibrium constant of the reaction between diphosphopyridine nucleotide and β -hydroxybutyrate

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

was determined by using D(-)- β -hydroxybutyric dehydrogenase from *Rhodopseudomonas spheroides* and found to be 1.42×10^{-9} at 25° . It is calculated from this value and the redox potential of the diphosphopyridine nucleotide system that the redox potential of the β -hydroxybutyrate-acetoacetate system is -0.266 v (pH 7.0, 25°).

We are indebted to Dr D. W. W. Henderson, F.R.S., and Mr R. Elsworth of the Microbiological Research Establishment, Porton, Wilts., for supplying bulk quantities of *Rhodopseudomonas spheroides*. This work was aided by grants from the Rockefeller Foundation and from the National Institutes of Health, United States Public Health Service.

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Effects of Iron Deficiency and of Zinc Deficiency on the Composition of *Mycobacterium smegmatis*

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Certain cultures of *Mycobacterium smegmatis* in Proskauer & Beck medium grew poorly and showed abnormalities in composition (Winder & Denny, 1956). It was found that this condition resulted from a deficiency of both iron and zinc in some batches of this medium, which relies on chance contamination of its constituents to provide these metals. Bacteria from these doubly deficient cultures had a low ribonucleic acid and deoxyribonucleic acid concentration from the time of growth inhibition, and possibly preceding it (Winder & Denny, 1959).

It has been known for some time that mycobacteria require iron (Sauton, 1912) and zinc (Drea, 1956) for growth. Both metals are probably essential for all organisms and each has been shown to be a component of a number of enzymes (Nicholas, 1957; Granick, 1958; Hoch & Vallee, 1958; Patwardhan, 1960; Kupiecki & Coon, 1960). Almost all of these enzymes are concerned with oxidation-reduction processes.

This paper is concerned with a comparison between the changes in amounts of some important components in *M. smegmatis* which take place when