An Enzymatic Assay Method for D(−)-3-Hydroxybutyrate and Acetoacetate Involving Acetoacetyl Coenzyme A Synthetase from *Zoogloea ramigera*

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An enzyme assay method for D(−)-3-hydroxybutyrate and acetoacetate involving acetoacetyl coenzyme A (CoA) synthetase was developed. To determine the concentration of D-3-hydroxybutyrate, it was oxidized with D-3-hydroxybutyrate dehydrogenase in the presence of nicotinamide adenine dinucleotide (NAD^+) to acetoacetate, which was then converted to acetyl CoA *via* acetoacetyl CoA through the combined actions of acetoacetyl CoA synthetase and 3-ketothiolase in the presence of adenosine triphosphate (ATP) and CoA. To determine the concentration of acetoacetate, acetoacetyl CoA generated from acetoacetate with acetoacetyl CoA synthetase was reduced to 3-hydroxybutyryl CoA with 3-hydroxyacyl CoA dehydrogenase in the presence of NADH. The amount of D-3-hydroxybutyrate or acetoacetate was estimated from the increase or decrease in the absorbance at 340 nm, respectively.

The present assay method seemed to be accurate and quick. Furthermore, as to the assaying of D-3-hydroxybutyrate, the omission of hydrazine, which is included for the standard method, may be preferable for routine assaying.

Keywords D(−)-3-hydroxybutyrate; acetoacetate; acetoacetyl CoA synthetase; ketone body; hydrazine; enzymatic assay; *Zoogloea ramigera*

D(−)-3-Hydroxybutyrate and acetoacetate, which are well known as ketone bodies in mammals, are formed in the liver and utilized by peripheral tissues as an energy source and a substrate for fatty acid and cholesterol biosynthesis.[2)] Since the concentration of ketone bodies in the blood is significantly affected by various physiological and pathological conditions in mammals, being elevated, for example, under starvation and diabetic conditions, it is desirable to have a reliable clinical assay method. The most commonly used assay method for D-3-hydroxybutyrate is the one described by Williamson *et al*,[3)] which involves D-3-hydroxybutyrate dehydrogenase purified from *Rhodopseudomonas spheroides*. Since the equilibrium constant for the reaction catalyzed by this enzyme [D(−)-3-hydroxybutyrate + nicotinamide adenine dinucleotide (NAD^+) $\rightleftharpoons$ acetoacetate + reduced nicotinamide adenine dinucleotide (NADH) + H^+] is 1.42×10^{-9},[4)] hydrazine is added to the reaction mixture at a relatively high concentration to shift the reaction equilibrium toward acetoacetate production through its hydrazone formation. However, hydrazine, which was reported to be carcinogenic in rats and mice,[5)] is not suitable for use in routine clinical assays. To overcome this drawback, Brashear and Cook[6)] developed a hydrazine-independent enzymatic assay for D-3-hydroxybutyrate, in which a higher pH (9.5 *vs.* 7.5) and a higher concentration of NAD^+ (15 mM *vs.* 1 mM) are used to shift the equilibrium of the enzymatic reaction in the direction of acetoacetate. In this report, we described a hydrazine-independent enzymatic assay method for ketone bodies involving acetoacetyl coenzyme A (CoA) synthetase (acetoacetate: CoA ligase, EC 6.2.1.16). We used *Zoogloea ramigera*, a floc-forming bacterium isolated from activated sludge which has a strong activity of acetoacetyl CoA synthetase[7)] as an enzyme source.

Experimental

Animals Male rats of the Wistar strain were maintained on standard laboratory feed or starved for 24 or 48 h. To obtain diabetic rats, a single dose of alloxan (200 mg/kg) was injected intraperitoneally.

Materials The materials used were obtained from the following sources: adenosine triphosphate (ATP), Sigma Chemical Co. (St. Louis, MO, U.S.A.); CoA, Kyowa Hakko Co. (Tokyo, Japan); 3-hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides*, 3-hydroxyacyl CoA dehydrogenase from pig heart and pyrophosphatase from yeast, Boehringer (Mannheim, FRG). Acetoacetyl CoA synthetase,[7)] 3-ketothiolase[8)] and D-3-hydroxybutyrate dehydrogenase[9)] were purified from *Zoogloea ramigera* I-16-M, as described previously. D(−)-3-Hydroxybutyrate was prepared by hydrolysis of poly(3-hydroxybutyrate). Lithium acetoacetate was prepared by hydrolysis of ethyl acetoacetate with lithium hydroxide followed by recrystallization.[10)] The concentration of the standard D-3-hydroxybutyrate and acetoacetate was determined chemically and enzymatically,[3)] respectively. Other chemicals of reagent grade were purchased from commercial sources.

Assaying of D-3-Hydroxybutyrate and Acetoacetate The principles of the assaying of these two compounds are shown in Chart 1. (A) To determine the concentration of D-3-hydroxybutyrate, it is first converted through the action of D-3-hydroxybutyrate dehydrogenase in the presence of NAD^+ to acetoacetate removed eventually as acetyl CoA *via* acetoacetyl CoA through the combined actions of acetoacetyl CoA synthetase and 3-ketothiolase in the presence of ATP and CoA. (B) For the assaying of acetoacetate, acetoacetyl CoA generated from acetoacetate is reduced to 3-hydroxybutyryl CoA with 3-hydroxyacyl CoA dehydrogenase in the presence of NADH. Pyrophosphatase is included to shift the equilibrium toward acetoacetyl CoA formaton.

(A) The assay mixture for D-3-hydroxybutyrate (0.75 ml) contained 100 mM Tris–HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM CoA, 0.67 mM NAD^+, 0.28 unit of acetoacetyl CoA synthetase, 0.13 unit of 3-ketothiolase and a sample. The initial absorbance of the assay mixture at 340 nm was determined with a Shimadzu recording spectrophotometer, model UV-240, after the absorbance had become constant. D-3-Hydroxybutyrate dehydrogenase (0.32 unit) was then added to the mixture, and the absorbance at 340 nm was monitored until it became constant. The increase in absorbance at 340 nm was used to calculate the amount of D-3-hydroxybutyrate in the sample.

(B) The assay mixture for acetoacetate (0.75 ml) contained 100 mM Tri–HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.13 mM NADH, 0.12 unit of acetoacetyl CoA synthetase, 0.66 unit of 3-hydroxyacyl CoA dehydrogenase, 0.75 unit of pyrophosphatase and a sample. After the initial absorbance of the assay mixture at 340 nm had been determined, 0.375 mM CoA was added, and then the absorbance was monitored until it became constant. The amount of acetoacetate in the sample was determined from the decrease in absorbance at 340 nm.

Blood samples for the determination of ketone bodies were prepared as described by Williamson *et al.*[3)] Ice-cold 30% (w/v) perchloric acid (300 μl) was added to 300 μl 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. The mixture was kept on ice for 30 min, and the insoluble potassium perchlorate centrifuged off. The determination of ketone bodies was carried

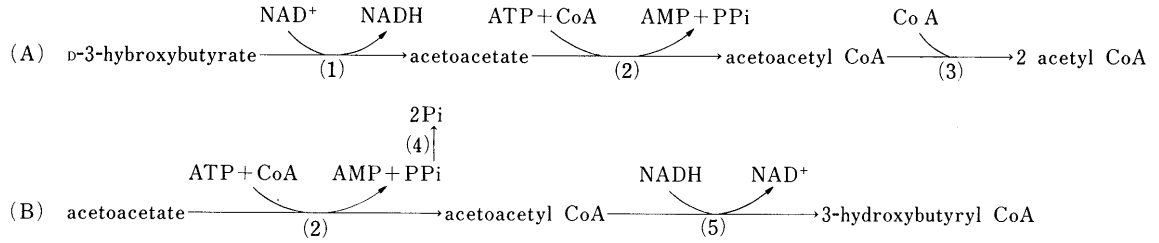

Chart 1. Principle of the New Enzymatic Assay for Ketone Bodies

(A) D-3-hydroxybutyrate and (B) acetoacetate. (1) D-3-hydroxybutyrate dehydrogenase, (2) acetoacetyl CoA synthetase, (3) 3-ketothiolase, (4) pyrophosphatase and (5) 3-hydroxyacyl CoA dehydrogenase.

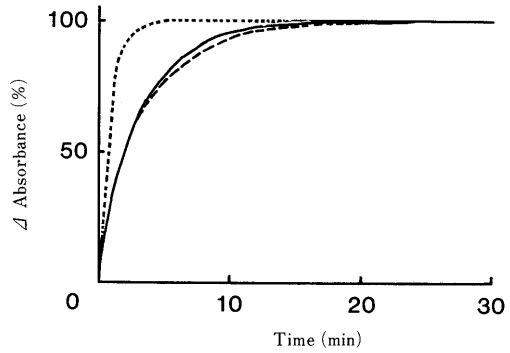

Fig. 1. Comparison of the Time Courses for Three Different Assay Methods for D-3-Hydroxybutyrate

D-3-Hydroxybutyrate (0.1 mM) was assayed enzymatically by the present method (------); at pH 9.5 (——) (5); and in the presence of hydrazine hydrate (– – –) (2) with the same number of units (0.32 unit) of D-3-hydroxybutyrate dehydrogenase.

TABLE I. Effect of Hydrazine and Other Factors on the Kinetic Parameters of the Reaction of D-3-Hydroxybutyrate Dehydrogenase

Enzyme source	Addition	K_m for D-3-hydroxybutyrate (mM)	V_{max} (percent of control)
Z. ramigera	None (control, pH 8.5)	0.38	(100)
	Hydrazine (0.33 M)	1.05	106
	NaCl (0.2 M)	1.25	129
	(0.5 M)	2.50	129
	pH 9.5	2.85	79
R. spheroides	None (control, pH 8.5)	0.48	(100)
	Hydrazine (0.33 M)	1.25	108
	pH 9.5	1.82	70

The activities of the D-3-hydroxybutyrate dehydrogenase (0.04 unit for *Z. ramigera* enzyme, 0.064 unit for *R. spheroides* enzyme) were measured under standard assay conditions in the absence (control; 50 mM Tris–HCl/pH 8.5) or presence of hydrazine hydrate (0.33 M) and NaCl (0.2 and 0.5 M), or at pH 9.5 (50 mM, glycine–NaOH). The K_m and V_{max} values were calculated from the initial velocities of the enzyme reactions.

out on the resulting supernatant.

TABLE II. Analytical Recovery and Precision of D-3-Hydroxybutyrate and Acetoacetate Assays

Ketone body (μM)	Measured concentration (μM)	Mean recovery (%)	Precision (C.V.) (%)
D-3-Hydroxybutyrate			
20	20.0		
	18.4		
	21.3		
	20.7		
	17.9		
	19.1		
	21.6	(99)	(6.6)
100	100.3		
	91.7		
	99.7		
	96.6		
	98.9		
	90.6		
	94.8	(96)	(3.5)
200	182		
	185		
	183		
	173		
	184		
	186		
	176	(91)	(2.5)
Acetoacetate			
13.3	16.6		
	14.9		
	14.9		
	17.2		
	12.1		
	13.8		
	13.2	(110)	(4.5)
133	122		
	124		
	117		
	126	(92)	(2.8)

The standard ketone bodies at several concentrations were added to the standard assay mixture. The recovery and the precision were calculated from the measured values of ketone bodies.

Results and Discussion

Reaction Time and Linearity of the Assay The reactions during the assaying of D-3-hydroxybutyrate (0.04—0.16 mM) and acetoacetate (0.013—0.107 mM) reached plateaus within 3—10 min at 30 °C, and the concentrations of D-3-hydroxybutyrate and acetoacetate in the samples were proportional to the changes in absorbance at 340 nm in the ranges of up to 0.2 and 0.1 mM, respectively (data not shown).

In the assaying of D-3-hydroxybutyrate, its quantitative conversion to acetoacetate with D-3-hydroxybutyrate is difficult unless acetoacetate is removed effectively. With both the present and hydrazine-based methods, the presence of even a 5-fold higher concentration of acetoacetate than of D-3-hydroxybutyrate in the sample did not interfere with the assaying of the latter (data not shown). Similarly, the presence of D-3-hydroxybutyrate did not interfere with the assaying of acetoacetate by the present method, and 95% of the total acetoacetate could be accounted for even in the presence of a 10-fold higher concentration of D-3-hydroxybutyrate than that of acetoacetate in sample (data not shown).

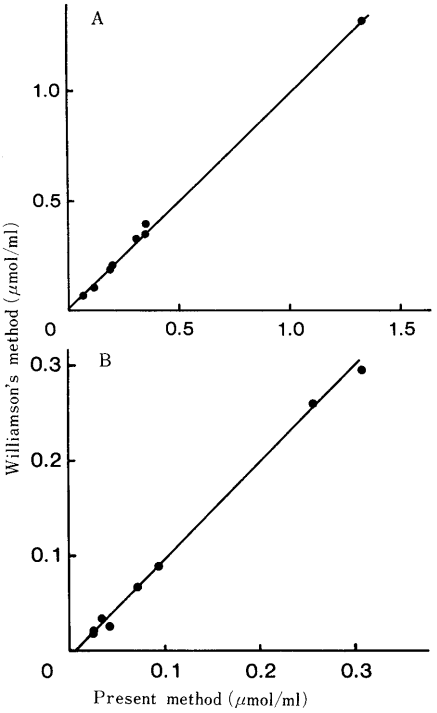

Fig. 2. Correlation between the Results Obtained with the Standard (Williamson's[3]) and the Present Enzymatic Assay Methods for D-3-Hydroxybutyrate (A) and Acetoacetate (B)

The concentrations of D-3-hydroxybutyrate and acetoacetate in blood samples from normal, starved and diabetic rats were assayed as described under Experimental. (A) $n=8$, $y=0.985x+0.0119$, $r=0.999$. (B) $n=8$, $y=1.018x-0.0098$, $r=0.996$.

Time Course of D-3-Hydroxybutyrate Assays Figure 1 shows the time courses of D-3-hydroxybutyrate assays with three different methods (the present, hydrazine- and pH-methods) using the same number of units of D-3-hydroxybutyrate dehydrogenase. The present method is the quickest, because one D-3-hydroxybutyrate assay (0.1 mM) could be completed within 5 min as compared with the more than 20 min required for the hydrazine- and pH 9.5-methods.

Since these results suggested possible inhibition of D-3-hydroxybutyrate dehydrogenase by hydrazine and a high pH, their effects on the kinetic parameters of the enzyme reaction were examined. As shown in Table I, the K_m value for D-3-hydroxybutyrate of the dehydrogenase became higher in the presence of hydrazine hydrate or pH 9.5 than the control value in the absence of this reagent (pH 8.5). A similar inhibitory effect was observed for D-3-hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides*, obtained commercially. Furthermore, high concentrations of NaCl (0.2—0.5 M) also increased the K_m value of D-3-hydroxybutyrate dehydrogenase from *Z. ramigera*. Therefore, the increase in the K_m value due to hydrazine hydrate may be attributable to the high ionic concentration (0.33 M).

Analytical Recovery and Precision The analytical recovery and precision (C.V.) of D-3-hydroxybutyrate and acetoacetate assay were shown in Table II. The lowest concentration of both ketone bodies detectable by the present method was about 5 μM. The upper limits of the assay of D-3-hydroxybutyrate and acetoacetate were about 0.3 and 0.1 mM, respectively. The following reagents did not interfere with the assay for both ketone bodies; ethylenediaminetetraacetic acid (EDTA) (5 mM), citrate (10 mM), malate (10 mM), succinate (10 mM) and heparin (100 unit/ml).

Correlation of the Present and the Standard Methods As shown in Fig. 2A and B, on the assaying of both D-3-hydroxybutyrate and acetoacetate, the results obtained with the present method showed good correlation with those obtained with the standard methods.[3]

The results obtained in the present study clearly show that our assay method for ketone bodies involving acetoacetyl CoA synthetase is accurate and quick. Furthermore, as to the assaying of D-3-hydroxybutyrate, the omission of hydrazine, a possible carcinogen, will make our assay method preferable for routine clinical assays.

Nowadays, as a clinical automated method for measuring ketone bodies in serum or urine, an enzymic assay method using an lactate dehydrogenase inhibitor (oxamic acid)[11] is widely used. This method seems to be quick and convenient for handling many samples, but metabolites other than pyruvate may influence the result and therefore it is probably not suited for the assay of ketone bodies in tissues. Although the assay method presented here is rather complicated and needs a special enzyme, acetoacetyl CoA synthetase, it is accurate, specific, and applicable to any samples.

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