# Inhibition of D(−)-β-Hydroxybutyrate Dehydrogenase by Butanedione, Phenylglyoxal, and Diethyl Pyrocarbonate†

Donna C. Phelps‡ and Youssef Hatefi*

ABSTRACT: D(−)-β-Hydroxybutyrate dehydrogenase of beef heart mitochondria is inhibited by modifiers of disulfides, thiols, and vicinal dithiols [Phelps, D. C., & Hatefi, Y. (1981) *Biochemistry 20* (preceding paper in this issue)]. The vicinal dithiol can be reversibly oxidized by diamide, resulting in activity inhibition, and rereduced by dithiothreitol, resulting in reactivation. The diamide-treated enzyme can no longer be irreversibly inhibited by *N*-ethylmaleimide, indicating the absence of an essential sulfhydryl group other than the vicinal dithiol. β-Hydroxybutyrate dehydrogenase also appears to contain essential arginyl residues modifiable by phenylglyoxal or butanedione, and essential residue(s) modifiable at pH 6.0 by diethyl pyrocarbonate. Substrates protect against inhibitions by butanedione, phenylglyoxal, and diethyl pyrocarbonate, suggesting that the essential, modifiable residues are at or near the substrate binding sites. On the basis of these results and pH profiles, tentative mechanisms have been proposed for the oxidation of β-hydroxybutyryate and the reduction of acetoacetate, involving the participation of the essential residues described above.

It has been shown in the preceding paper of this issue (Phelps & Hatefi, 1981) that D(−)-β-hydroxybutyrate dehydrogenase of beef heart mitochondria is inhibited by modifiers of thiols, vicinal dithols, and disulfides. Presence of an essential dithiol in the enzyme was suggested by inhibitory effects of NEM,[1] *p*CMS, diamide, and phenylarsine oxide. NAD and NADH, but not β-hydroxybutyrate and acetoacetate, protected the enzyme against inhibition by these mono- and dithiol modifiers. Inhibition by sulfite and sulfide suggested either the modification of a disulfide or adduct formation between NAD and sulfite or sulfide followed by formation of an inhibited complex involving the NAD adduct and enzyme.

The present communication presents data on the inhibition of β-hydroxybutyrate dehydrogenase by butanedione, phenylglyoxal, and diethyl pyrocarbonate. The residues modified by these reagents appear to be at or near the substrate binding sites of the enzyme, since the presence of appropriate substrates protects the enzyme against inhibition. These findings will be discussed in relation to the mechanism of action of β-hydroxybutyrate dehydrogenase.

## Materials and Methods

Submitochondrial particles were prepared and protein concentrations determined as in the preceding paper of this issue (Phelps & Hatefi, 1981). Except for the details specified in the figure legends, the assays for β-hydroxybutyrate oxidation and acetoacetate reduction were also the same as before (Phelps & Hatefi, 1981). Specific activity is expressed as micromoles of NAD reduced or NADH oxidized per minute per milligram of protein at 36–37 °C.

Phenylglyoxal and butanedione were obtained from Aldrich Chemical Co., and diethyl pyrocarbonate was obtained from Sigma Chemical Co. The sources of other chemicals were the same as before (Phelps & Hatefi, 1981).

## Results

As stated above, it has been shown in the preceding paper of this issue that the mitochondrial D(−)-β-hydroxybutyrate dehydrogenase contains as essential dithiol and possibly a disulfide, both apparently involved in substrate binding. Since modifiers of monothiols (NEM, *p*CMS) and dithiols (diamide, phenylarsine oxide) both inhibited the enzyme, it was of interest to know whether (a) the essential monothiol was part of the essential dithiol, and (b) the dithiol and the disulfide (if at all present) were separate entities or one of the sulfhydryls of the dithiol was produced from substrate interaction with the presumed essential disulfide.

*Effect of NEM on Diamide-Inhibited Particles.* The points raised above were checked by the experiments described in Figure 1 for β-hydroxybutyrate oxidation (forward reaction) and acetoacetate reduction (reverse reaction). In each case, submitochondrial particles in the absence of added substrates were divided into three parts, A, B, and C. Aliquot A was treated with diamide which oxidizes vicinal dithiols to disulfides, aliquot B was treated with NEM which irreversibly inhibits monothiols, and aliquot C was treated with the inhibitor solvent, i.e., ethanol, each for 10 min at 0 °C. Then each aliquot was diluted 16-fold with 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.5, and centrifuged for 30 min at 100000*g* in the Spinco Model L ultracentrifuge. Supernatants were discarded, tubes were rinsed, and pellets were taken up in 0.25 M sucrose containing 50 mM Tris-HCl, pH 8.1, homogenized, and adjusted to 10 mg of protein/mL. Aliquot A was divided into two parts; one part was treated with NEM and the other with the same volume of ethanol. Aliquot B was also divided into two parts; one part was treated with diamide and the other with the same volume of ethanol. The control aliquot C was divided into three parts; one part was treated with NEM, the second with diamide, and the third again with ethanol. The mixtures were incubated for 10 min on ice and assayed for activity. Then 20 mM 1,4-dithiothreitol was added to each tube, and the mixtures were incubated 60–70 min on ice and assayed again for activity. The results for both the forward and reverse reactions showed that initial treatment with NEM inhibited the enzyme irreversibly.

† From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received May 21, 1980.* This work was supported by U.S. Public Health Service Grant AM 08126.

‡ D.C.P. was supported by U.S. Public Health Service Training Grant AM 07097.

[1] Abbreviations used: NEM, *N*-ethylmaleimide; *p*CMS, *p*-(chloromercuri)phenylsulfonate; diamide, diazenedicarboxylic acid bis(dimethylamide); SMP, submitochondrial particles; AcPyAD and AcPyADH, oxidized and reduced 3-acetylpyridine adenine dinucleotide, respectively; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/81/0420-0459$01.00/0 

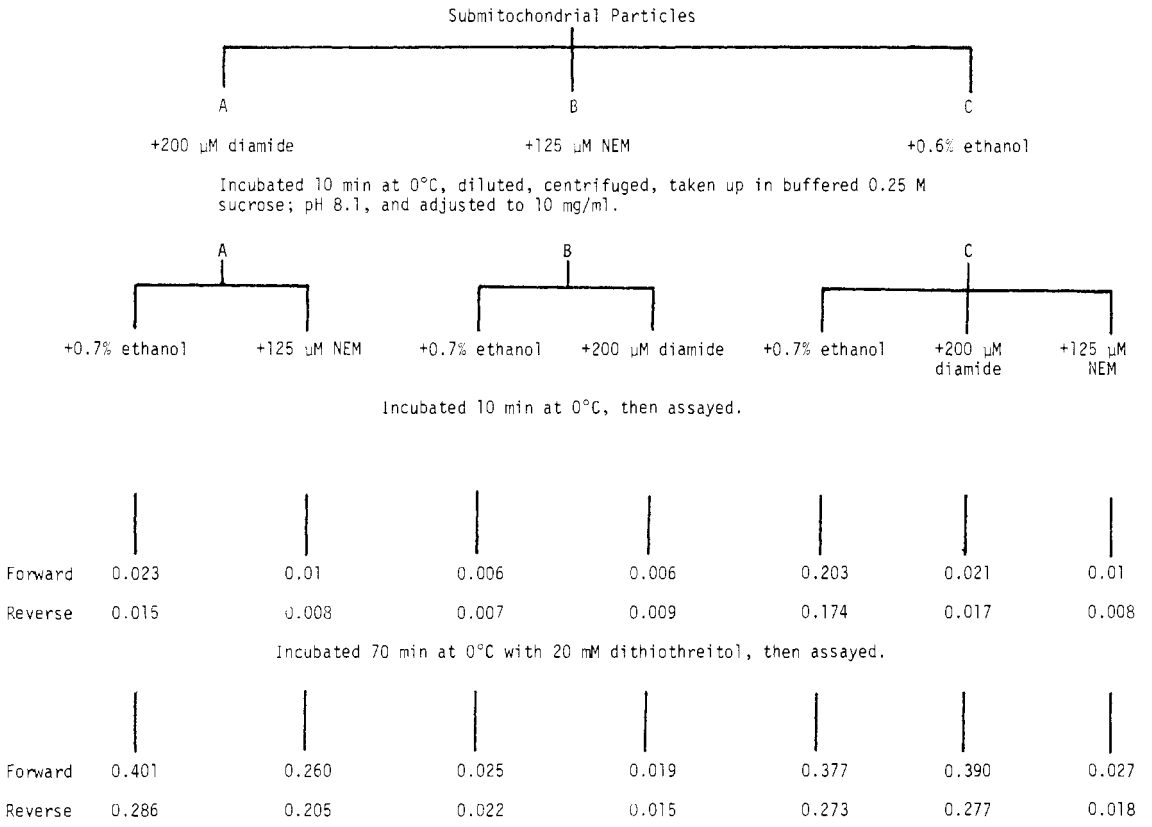


FIGURE 1: Protection of β-hydroxybutyrate dehydrogenase by diamide treatment against irreversible inhibition by NEM. Submitochondrial particles were originally suspended at 9.15 mg/mL in 0.25 M sucrose containing 50 mM Tris-HCl, pH 8.1. Other conditions have been described in the text. Forward and Reverse refer to rates in micromoles per minute per milligram of protein at 36 °C obtained for each fraction for β-hydroxybutyrate oxidation and acetoacetate reduction, respectively.

Treatment with diamide alone inhibited the enzyme, but activity could be recovered by subsequent treatment of the particles with dithiothreitol. The same was true when the diamide-inhibited particles were subsequently treated with NEM and finally with dithiothreitol. These results indicate the following: (a) In the absence of added substrates, β-hydroxybutyrate dehydrogenase contains a vicinal dithiol susceptible to oxidation by diamide to a disulfide. This oxidation inhibits the enzyme but can be reversed by treating the diamide-inhibited enzyme with dithiothreitol, which presumably converts the diamide-oxidized disulfide back to dithiol. (b) The NEM-modified, essential sulfhydryl group(s) is a component of the vicinal dithiol, because diamide-treated particles could not be irreversibly inhibited by treatment with NEM, and could be revived after NEM treatment by the addition of excess dithiothreitol (Figure 1). These results also indicate that the enzyme does not contain a separate essential sulfhydryl group in addition to the vicinal dithiol. Thus, since the monomer of β-hydroxybutyrate dehydrogenase has been shown by Bock & Fleischer (1975) to contain 5–6 cysteine residues per mol, it appears that two cysteine residues are at the active site as a vicinal dithiol. The remaining cysteine residues are either not modifiable by the above reagents (e.g., they could exist as a disulfide) or are not essential for catalytic activity of the enzyme.

As shown in Figure 1, treatment with dithiothreitol not only reversed diamide inhibition, but when no NEM was added it even improved the activity of the particles. This is probably due to rereduction of some of the vicinal dithiols that might have become air oxidized during preparation and storage of the particles. However, as discussed above, β-hydroxybutyrate dehydrogenase contains not only an essential dithiol but also possibly an essential disulfide as well. Therefore, if modification of the disulfide results in inhibition, we would have to assume that this disulfide was either not reducible by dithiothreitol under the conditions used above (see Figure 1) or rapidly oxidized when the particles were added to the assay mixture. However, a much stronger possibility is that the inhibition by sulfite and sulfide is due to adduct formation with NAD (Hug et al., 1978; Parker et al., 1978) and not because of disulfide modification.

*Effects of Phenylglyoxal and Butanedione.* A large number of nicotinamide nucleotide linked enzymes are now known to contain arginyl residues at the active site, apparently serving as positively charged loci for recognition of the negatively charged phosphoryl moieties of the nucleotide coenzymes. Our previous studies have revealed the presence of three essential arginyl residues associated with mitochondrial inner membrane enzymes (Djavadi-Ohaniance & Hatefi, 1975; Frigeri et al., 1977, 1978). The possibility of the presence of arginyl or lysyl residues at the active site of β-hydroxybutyrate dehydrogenase was, therefore, considered not only because this enzyme is NAD linked but also because of our finding that DL-β-hydroxybutyryl ethyl ester did not serve as a substrate, suggesting the involvement of the negatively charged carboxyl group of β-hydroxybutyrate in binding. The results summarized in Figures 2 and 3 showed that indeed both phenylglyoxal and butanedione are potent inhibitors of β-hydroxybutyrate oxidation. As with most enzyme systems, phenylglyoxal was a stronger inhibitor and, as seen in Figure 2, it inhibited the enzyme so rapidly that it was difficult to follow the initial course of inhibition. However, with the less potent inhibitor, butanedione, the semilogarithmic plots of activity as a function of time showed a rapid initial rate of inhibition followed by a slower rate at several concentrations of the inhibitor (Figure 3). These results suggested the presence of more than one modifiable arginyl residue in β-hydroxybutyrate dehydrogenase. Analysis of the data of Figure 3 also indicated

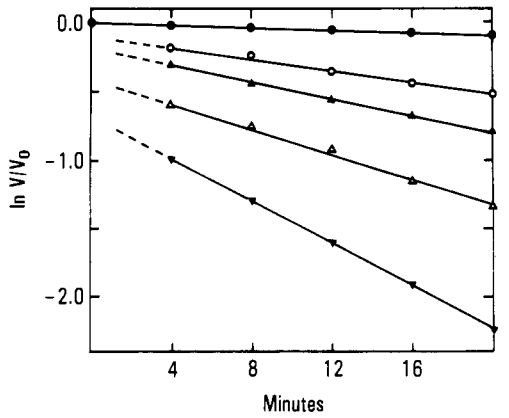


FIGURE 2: Semilogarithmic plots of the inhibition time course of β-hydroxybutyrate dehydrogenase activity at 0 (●), 1 (○), 2.2 (▲), 4.6 (△), and 10 (▼) mM phenylglyoxal. SMP at 5 mg of protein/mL of 0.12 M sucrose containing 50 mM Hepes/NaOH, pH 8.1, was incubated at 20 °C in the absence or presence of phenylglyoxal as indicated. At the intervals shown, 20-μL aliquots were removed and added to an assay mixture at 36 °C containing in 0.98 mL of 50 mM Hepes/Tris, pH 8.1, 5 μM rotenone, 10 mM DL-β-hydroxybutyrate, and 1 mM NAD. $V_0$, specific activity in micromoles of NAD reduced per minute per milligram of protein at time zero; $V$, specific activity at the times shown on the abscissa.

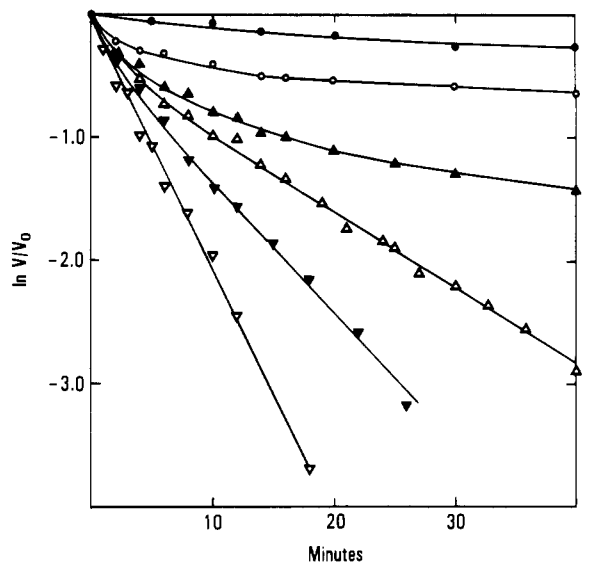


FIGURE 3: Semilogarithmic plots of the inhibition time course of β-hydroxybutyrate dehydrogenase activity at 0 (●), 10 (○), 20 (▲), 30 (△), 40 (▼), and 50 (▽) mM butanedione. Submitochondrial particles at 5 mg of protein/mL of 0.12 M sucrose containing 5 μM rotenone, 50 mM borate, and 20 mM Tris, pH 8.1, were incubated at 20 °C in the absence or presence of butanedione as indicated. At the intervals shown, 20-μL aliquots were removed and assayed for activity as in Figure 2. $V_0$ and $V$ are the same as in Figure 2.

that the initial rapid phase of inhibition was much less affected by inhibitor concentration than the second phase.

When SMP was incubated with butanedione in the presence of β-hydroxybutyrate, NAD, or acetoacetate, each substrate alone had essentially no effect on the inhibition brought about by butanedione, while the presence of NADH increased the butanedione effect (Figure 4). However, the presence of a nucleotide plus β-hydroxybutyrate or acetoacetate in the incubation mixture offered considerable protection against inhibtion by butanedione. In order to study the effect of the substrates of the forward and the reverse reactions without formation of substantial amounts of products during the course of incubation of the particles with butanedione, NAD and NADH were replaced by the less reactive 3-acetylpyridine analogues, namely, AcPyAD and AcPyADH. The apparent

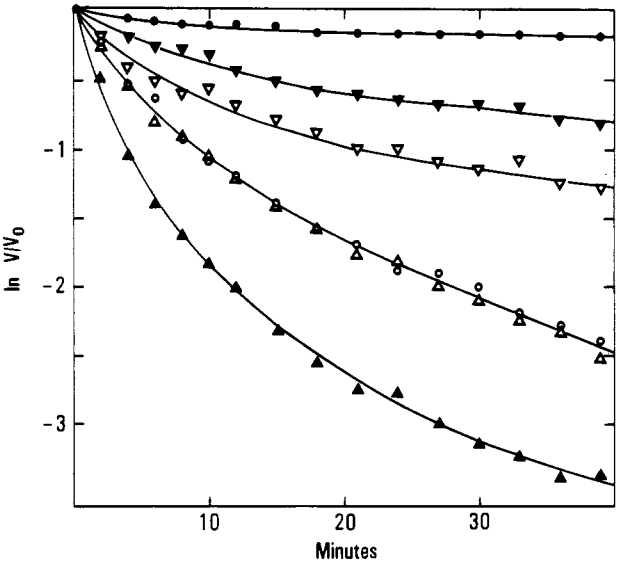


FIGURE 4: Semilogarithmic plots of the inhibition time course of β-hydroxybutyrate dehydrogenase activity as affected by incubation of SMP with 2 mM NAD, 1 mM NADH, or 10 mM β-hydroxybutyrate (●); 30 mM butanedione (○); 30 mM butanedione plus 2 mM NAD (△); 30 mM butanedione plus 1 mM NADH (▲); 30 mM butanedione plus 10 mM DL-β-hydroxybutyrate and 3 mM AcPyAD (▼); 30 mM butanedione plus 2 mM acetoacetate and 3 mM AcPyADH (▽). Conditions were the same as in Figure 3. Substrates were added to the 20 °C incubation mixture immediately before addition of butanedione where indicated. $V_0$ and $V$ are the same as in Figure 2.

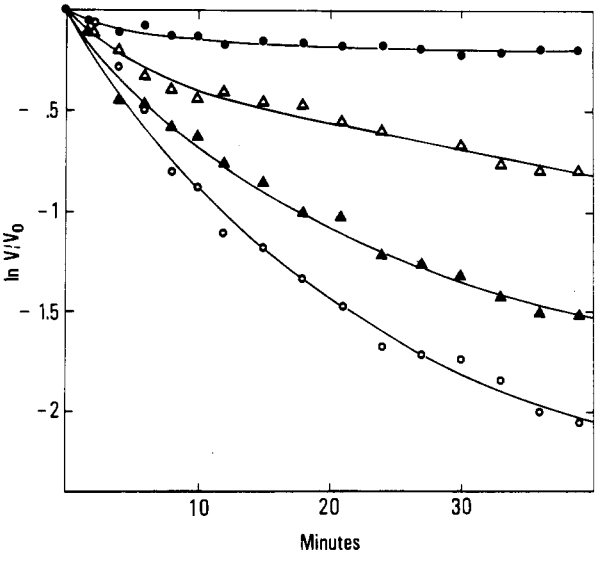


FIGURE 5: Semilogarithmic plots of the inhibition time course of β-hydroxybutyrate dehydrogenase activity in the absence of butanedione ± substrates (●) or in the presence of 30 mM butanedione (○) plus 10 mM DL-β-hydroxybutyrate and 3 mM AcPyADH (▲), or 2 mM acetoacetate plus 3 mM AcPyAD (△). Conditions were the same as in Figure 4. $V_0$ and $V$ are the same as in Figure 2.

$K_m$ of AcPyAD in the forward reaction was found to be 0.28 mM, which is not too different from the $K_m$ for NAD (0.17 mM; Phelps & Hatefi, 1981). However, the apparent $V_{max}$ in the presence of AcPyAD was only about 0.02 μmol $min^{-1}$ $(mg\ of\ protein)^{-1}$, i.e., about 25 times less than the $V_{max}$ in the presence of NAD. Thus, with the use of the 3-acetylpyridine analogues, it was found that incubation of SMP with butanedione in the presence of β-hydroxybutyrate plus AcPyAD or of acetoacetate plus AcPyADH resulted in considerable protection (Figure 4). Not only the combination of productive substrates offered protection, but as seen in Figure 5 the presence of two reduced substrates, i.e., β-hydroxybutyrate plus

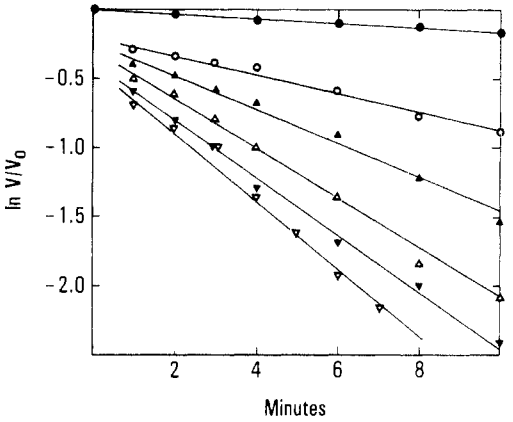


FIGURE 6: Semilogarithmic plots of the inhibition time course of β-hydroxybutyrate dehydrogenase activity at 0 (●), 1.6 (○), 2.4 (▲), 3.2 (△), 4.0 (▼) and 5 (▽) mM diethylpyrocarbonate. Submitochondrial particles at 0.5 mg/mL of 20 mM sodium 2-(*N*-morpholino)ethanesulfonic acid, pH 6.0, and 5 μM rotenone were incubated in the absence or presence of diethyl pyrocarbonate as indicated. At the intervals shown, 100-μL aliquots were removed and added to 0.9 mL of assay mixtures with the compositon given in Figure 2 for activity assay. $V_0$ and $V$ are the same as in Figure 2.

AcPyADH, or of two oxidized substrates, i.e., acetoacetate plus AcPyAD, also protected the enzyme against butanedione inhibition. The finding that a single substrate alone did not protect the enzyme against butanedione inhibition while the addition of both types of substrates, whether productive or not, afforded protection is consistent with the bi-bi mechanism of the enzyme (Nielsen et al., 1973), if we assume that the enzyme contains an arginyl residue concerned with β-hydroxybutyrate/acetoacetate binding and that NAD(H) must bind before the carboxylic acid can bind and protect.

*Effect of Diethyl Pyrocarbonate.* It was found by Latruffe & Gaudemer (1975) that diethyl pyrocarbonate inactivates the rat liver mitochondrial D(−)-β-hydroxybutyrate dehydrogenase at pH 6.1, suggesting to them the presence in the enzyme of an essential histidyl residue. As shown in Figure 6, the beef heart enzyme is also inhibited by diethyl pyrocarbonate at pH 6.0. In this case also, the semilogarithmic plots appeared to be biphasic, consisting of a rapid initial phase of inhibition followed by a second linear phase at the inhibitor concentrations examined. The effects of substrates on the inhibition of β-hydroxybutyrate dehydrogenase activity by diethyl pyrocarbonate are shown in Table I. it is seen that β-hydroxybutyrate, acetoacetate, or NAD alone had no effect; NADH protected, and so did the combinations of β-hydroxybutyrate or acetoacetate plus an oxidized or a reduced nucleotide. While rather specific at pH 6.0 for histidyl residues, diethyl pyrocarbonate could also react with other groups such as lysyl, tyrosyl, and arginyl residues. However, as regards arginyl residues, the data of Figure 4 and Table I show a clear difference in the effect of substrates on the inhibitory effects of butanedione and diethyl pyrocarbonate. As seen in Figure 4, NADH promoted the inhibition by butanedione, while as indicated in Table I, NADH offered considerable protection against the inhibitory effect of diethyl pyrocarbonate.

Table I: Substrate Protection of Inhibition of β-Hydroxybutyrate Oxidation by Diethyl Pyrocarbonate[a]

| additions | $k - k_0$ |
|---|---|
| none | 0.16 |
| 10 mM β-hydroxybutyrate | 0.15 |
| 2 mM acetoacetate | 0.16 |
| 2 mM NAD | 0.15 |
| 1 mM NADH | 0.06 |
| 10 mM β-hydroxybutyrate + 3 mM AcPyAD | 0.07 |
| 10 mM β-hydroxybutyrate + 3 mM AcPyADH | 0.04 |
| 3 mM acetoacetate + 3 mM AcPyAD | 0.10 |
| 3 mM acetoacetate + 3 mM AcPyADH | 0.06 |

[a] Conditions were the same as in Figure 6. $k$, the pseudo-first-order rate constants of the linear portion of semilogarithmic plots as shown in Figure 6 in the presence of 3.2 mM diethyl pyrocarbonate ± substrates as indicated; $k_0$ the pseudo-first-order rate constants in the absence of diethyl pyrocarbonate ± substrates as indicated.

## Discussion

The inhibition of β-hydroxybutyrate dehydrogenase by the arginine-specific reagents phenylglyoxal and butanedione suggests that the enzyme contains essential arginyl residues. The observation that a single substrate alone did not protect the enzyme against butanedione inhibition while β-hydroxybutyrate or acetoacetate in the presence of either NAD or NADH afforded considerable protection suggests further that at least one essential arginyl residue might be present at or near the binding site of β-hydroxybutyrate/acetoacetate. This is because according to the results of Nielsen et al. (1973) the kinetics of the enzyme are ordered bi-bi, with NAD being the first substrate to add in the forward reaction and NADH the last product to leave. Thus, binding of and protection by β-hydroxybutyrate/acetoacetate would require the presence of NAD(H). The above conclusion is also consistent with the observation that β-hydroxybutyryl ethyl ester did not serve as a substrate, suggesting that the negatively charged carboxyl group might be involved in substrate binding.

In addition to the above, we have shown that similar to the rat liver mitochondrial β-hydroxybutyrate dehydrogenase (Latruffe & Gaudemer, 1975), the beef heart enzyme is also inhibited by diethyl pyrocarbonate at pH 6.0, suggesting the involvement of essential histidyl residue(s). The diethyl pyrocarbonate modified residue(s) also appears to be at the enzyme active site because of the substrate protection effects shown in Table I. In this table, it was shown that except for NADH any substrate added alone did not afford protection, while addition of either carboxylic acid together with a nucleotide, even the nonproductive pair of acetoacetate plus AcPyAD, did. These results do not allow the conclusion that the protectable residue(s) is at or near the NAD(H) binding site, because NAD alone did not afford protection. However, they do permit a conclusion in favor of the carboxylic acids in accordance with the ordered bi-bi mechanism discussed above. With regard to protection by NADH alone, it may be recalled from Figure 4 that NADH also had a peculiar effect on inhibition of the enzyme by butanedione. In this case, it promoted butanedione inhibition while any other substrate alone had no effect. Considered together, these two opposing effects of NADH suggest the possibility that NADH binding might alter the availability of various residues at the enzyme active site to external reagents. If so, then why is it that NAD does not do the same? The answer to this question may be associated with the possibility that NADH binding shields a positively charged group at the active site. However, NAD while shielding the same charge introduces a positive charge of its own at the nicotinamide ring.

These details aside, we are impressed by the similarity of amino acid residues at the active sites of β-hydroxybutyrate dehydrogenase and lactate dehydrogenase (Holbrook et al., 1975). Both enzymes are NAD linked, β-hydroxybutyrate dehydrogenase appears to contain both arginyl and possibly histidyl residues at or near the active site, and lactate dehydrogenase histidyl residue 195 and arginyl residues 109 and 171 also are considered to be at or near its active site.

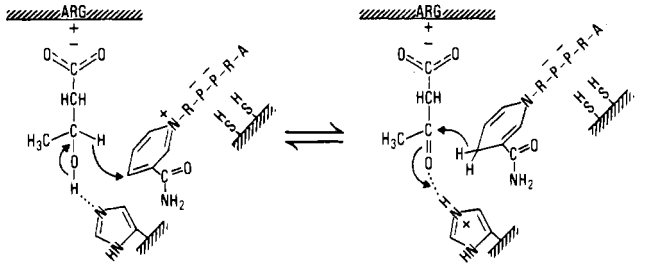

FIGURE 7: Scheme showing the possible roles of histidyl and arginyl rsidues in β-hydroxybutyrate oxidation (left-hand side) and acetoacetate reduction (right-hand side). R, P, and A are, respectively, the ribose, phosphate, and adenine moieties of NAD(H); ARG is the arginine residue, and shaded areas are portions of the β-hydroxybutyrate dehydrogenase enzyme.

Moreover, β-hydroxybutyrate and acetoacetate are analogues of lactate and pyruvate, the difference being that the former substrate pair is longer by one methylene group. Indeed, as shown by Latruffe & Gaudemer (1975) and ourselves, D-(−)-lactate is an inhibitor of β-hydroxybutyrate oxidation. Therefore, we feel that the arginyl and histidyl residues may serve similar functions at the active sites of these two enzymes. Thus, as discussed above, one arginyl residue might be involved in anchoring the carboxyl group of substrates [see also the similar suggestion of Holbrook et al. (1975) for Arg-171 in lactate dehydrogenase].

Should the diethyl pyrocarbonate modified residue prove to belong to histidine, then the electron-rich nitrogen of the imidazole ring might serve as a base (see Figure 7) for hydrogen bonding with the hydroxyl group of β-hydroxybutyrate. This arrangement would then labilize the $H^-$ group on the β carbon of the substrate. Holbrook et al. (1975) suggest a similar role for His-195 of lactate dehydrogenase. We further propose that in the reverse reaction, i.e., acetoacetate reduction, the protonated form of the nitrogen of the imidazole ring is the species that participates in interaction with the substrate. In this case $=\overset{|}{N}H^+$ would form a hydrogen bond with the β-carbonyl group of acetoacetate as shown in Figure 7, thus making the β carbon electrophilic and reducible. Support for this possible role of the presumed histidyl residue is found in the results of Latruffe & Gaudemer (1975) for the rat liver mitochondrial enzyme and in our own data for the beef heart enzyme. They showed that the pH optimum for β-hydroxybutyrate oxidation is 8.2, while for acetoacetate reduction it is around pH 7. Our results with the beef heart enzyme showed similar pH optima, i.e., 7.8–8.2 for the forward reaction and about one pH unit more acid for the reverse reaction. Since the pK of the imidazolium group of histidine in most proteins is below pH 7.0 (White et al., 1959), these results are consistent, therefore, with the possibility of the unprotonated form of an essential histidyl residue participating in β-hydroxybutyrate binding and oxidation, while its protonated form being the species required for acetoacetate binding and reduction (see Figure 7).

What are the roles of the essential dithiol and the phospholipid cofactor (Jurtshuk et al., 1963; Gazotti et al., 1974, 1975) in the mechanism of action of β-hydroxybutyrate dehydrogenase? The available data do not permit much speculation at this time. Both the dithiol (Phelps & Hatefi, 1981) and the phospholipid (Gazotti et al., 1974) appear to be concerned with nucleotide binding; however, whether they participate as functional groups in substrate reduction and oxidation has yet to be determined. Nonetheless, the data of the preceding (Phelps & Hatefi, 1981) and the present paper have shown the apparent existence of the following essential residues in β-hydroxybutyrate dehydrogenase: (a) a dithiol at or near the NAD(H) binding site, (b) one or more arginyl residues protectable against modification when both substrates are present, presumably involved in anchoring the negatively charged carboxyl group of β-hydroxybutyrate/acetoacetate, and (c) presumably a histidyl residue which in its unprotonated and protonated forms might be involved in hydrogen bonding, respectively, with the β-hydroxyl group of β-hydroxybutyrate and the β-carbonyl group of acetoacetate. Adding to this list the phospholipid cofacotr (apparently lecithin) discovered by others (Jurtshuk et al., 1963; Gazotti et al., 1974, 1975) and the inhibitory effects of sulfite, sulfide, and cyanide, the available information provides for an interesting challenge to determine the precise effects of these components and reagents at the active site and unravel the details of the mechanism of action of D(−)-β-hydroxybutyrate dehydrogenase.

Acknowledgments

We thank C. Munoz for the preparation of mitochondria.

References

Bock, H.-G., & Fleischer, S. (1975) *J. Biol. Chem. 250*, 5774–5781.

Djavadi-Ohaniance, L., & Hatefi, Y. (1975) *J. Biol. Chem. 250*, 9397–9403.

Frigeri, L., Galante, Y. M., Hanstein, W. G., & Hatefi, Y. (1977) *J. Biol. Chem. 252*, 3147–3152.

Frigeri, L., Galante, Y. M., & Hatefi, Y. (1978) *J. Biol. Chem. 253*, 8935–8940.

Gazotti, P., Bock, H.-G., & Fleischer, S. (1974) *Biochem. Biophys. Res. Commun. 58*, 309–315.

Gazotti, P., Bock, H.-G., & Fleischer, S. (1975) *J. Biol. Chem. 250*, 5782–5790.

Holbrook, J. J., Liljas, A., Steindel, S. J., & Rossman, M. G. (1975) *Enzymes, 3rd Ed. 11*, 191–292.

Hug, D. H., O'Donnell, P. S., & Hunter, J. K. (1978) *J. Biol. Chem. 253*, 7622–7629.

Jurtshuk, P., Jr., Sekuzu, I., & Green, D. E. (1963) *J. Biol. Chem. 238*, 3595–3605.

Latruffe, N., & Gaudemer, Y. (1975) *Biochimie 57*, 849–857.

Nielsen, N. C., Zahler, W. L., & Fleischer, S. (1973) *J. Biol. Chem. 248*, 2556–2562.

Parker, D. M., Lodola, A., & Holbrook, J. J. (1978) *Biochem. J. 173*, 959–967.

Phelps, D. C., & Hatefi, Y. (1981) *Biochemistry* (preceding paper in this issue).

White, A., Handler, P., Smith, E. L., & Stetten, DeW. (1959) in *Principles of Biochemistry*, p 139, McGraw-Hill, New York.