

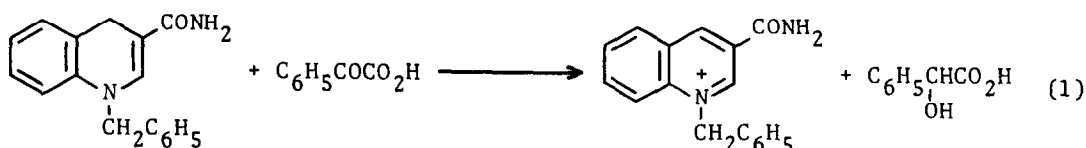
BIOMIMETIC REDUCTION OF BENZOYLFORMIC ACID BY AN ACID STABLE NADH  
ANALOGUE : A MODEL REACTION OF LACTATE DEHYDROGENASE

Seiji SHINKAI\*, Hisatake HAMADA, Yumiko KUSANO, and Osamu MANABE  
Department of Industrial Chemistry, Faculty of Engineering,  
Nagasaki University, Nagasaki 852, Japan

By the use of an acid-stable NADH analogue(3-carbamoyl-N-benzyl-1,4-dihydroquinoline) NADH model reduction of benzoylformic acid in aqueous solution was achieved, for the first time, at pH region where the carboxyl group is undissociated.

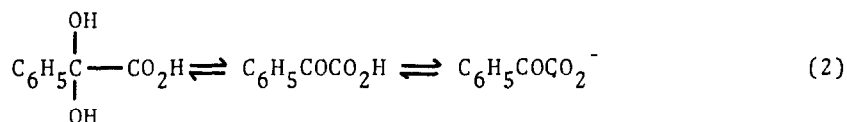
Lactate dehydrogenase is a NADH-dependent enzyme which catalyzes the interconversion of pyruvic acid and lactic acid coupled to the oxidation-reduction of NADH.<sup>1,2)</sup> While esters of  $\alpha$ -keto acids can be easily reduced nonenzymatically by 1,4-dihydropyridines(NADH model compounds) in acetonitrile with the aid of metal ions,<sup>3)</sup> reduction of  $\alpha$ -keto acids in aqueous solution which is a closely resembled model reaction of lactate dehydrogenase involves considerable difficulty because the substrates exhibit acidity strong enough to cause the rapid decomposition of 1,4-dihydropyridines.<sup>4,5)</sup> Thus, the information which is relevant to the oxidoreduction of lactate dehydrogenase has hardly been provided with certainty from a standpoint of model investigations.

Recently, we found that 1-benzyl-3-carbamoyl-1,4-dihydroquinoline(BzlQH), in which an acid-sensitive carbon-carbon double bond of the 1,4-dihydropyridine structure is protected by aromatic ring, is unusually stable against proton acids and thus becomes a useful model compound to investigate the NADH-dependent reactions in acidic aqueous solution.<sup>6)</sup> In this communication, we report the facile reduction of undissociated benzoylformic acid by BzlQH under ambient reaction conditions and discuss the biological implications in connection with the diagrammatically represented active site of lactate dehydrogenase.<sup>1,7)</sup>



In the first place, benzoylformic acid was subjected to the BzlQH reduction in a preparative scale, and the increase of mandelic acid with reaction time was confirmed by analysis with high-speed liquid chromatography.<sup>8)</sup> The kinetic and spectroscopic measurements were carried out at 50°C in 25 vol% aqueous isopropanol at a calculated ionic strength of 1.12. The reaction rates were estimated by following the decrease in the absorption band of BzlQH at 343 nm ( $\epsilon=13100$ ) and the rate data were corrected for the relatively slow spontaneous decay of BzlQH which occurred in the absence of benzoylformic acid.

The UV spectrum of benzoylformic acid in acetonitrile gave an absorption maximum at 253 nm ( $\epsilon=7780$ ). A similar absorption maximum also appeared in aqueous solution at 255 nm which is attributed to the absorption band of undissociated form of benzoylformic acid. The optical density (OD) increased with lowering medium pH by the addition of HCl and experienced a maximum value at pH 1.3 (apparent  $\epsilon=9480$ ). No isosbestic point was found. These results imply that no less than two equilibria exist at pH region 1 to 5: one would stem from dissociation of the carboxyl group and the other, probably, from hydration of the carbonyl group.<sup>9)</sup>



Although it is difficult to evaluate the fraction of  $\text{C}_6\text{H}_5\text{COCO}_2\text{H}$  quantitatively from the spectral data because of the lack of isosbestic point, the qualitative amount may be expressed by the OD value at around 255 nm. We tentatively chose  $\text{OD}_{270} - \text{OD}_{270}$  at pH 6.0 as a measure of the  $\text{C}_6\text{H}_5\text{COCO}_2\text{H}$  fraction and illustrated the pH dependence in Fig. 1, together with the apparent second-order rate constants,  $k_2'$  ( $=v_{\text{obs}}/[\text{BzlQH}][\text{benzoylformic acid}]$ ).

Examination of Fig. 1 reveals that (1) the  $k_2'$  values at pH > 4 were so small ( $1 \sim 2 \times 10^{-3} \text{ M}^{-1}\text{sec}^{-1}$ ) that  $\text{C}_6\text{H}_5\text{COCO}_2^-$  would be almost unreactive toward BzlQH, and (2) in contrast to the maximum value of  $\text{OD}_{270} - \text{OD}_{270}$  (pH 6.0) at low pH region, the  $k_2'$  values increased acceleratively with lowering medium pH and  $0.129 \text{ M}^{-1}\text{sec}^{-1}$  resulted at pH 1.17. The discrepancy between  $k_2'$  and OD at low pH region strongly suggests that BzlQH reduction of benzoylformic acid is subject to acid-catalysis as noticed in some related systems.<sup>6)</sup> Assuming the  $\text{OD}_{270} - \text{OD}_{270}$  (pH 6.0) to be almost constant at pH 1.17 to 1.58 ( $=0.80 \pm 0.02$ ), the  $k_2'$  value was plotted against  $[\text{H}^+]$  in Fig. 2. A linear correlation ( $r=0.997$ ) strongly supports that the reaction is acid-catalyzed and the  $k_2'$  is given by Eq. 3,<sup>6)</sup>

$$k_2' = k_0' + k_H' [H^+] \quad (3)$$

where  $k_0'$  is the second-order rate constant for the water-catalyzed term and  $k_H'$  is the third-order rate constant for the oxonium ion catalysis. By means of

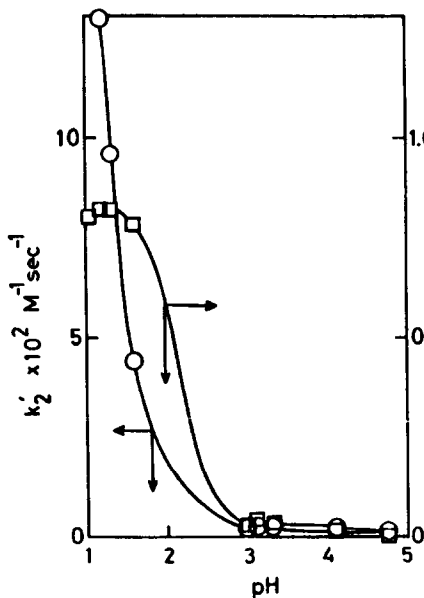


Fig. 1 Plots of  $k_2'$  and  $OD_{270} - OD_{270}$  at pH 6.0 as a function of pH.

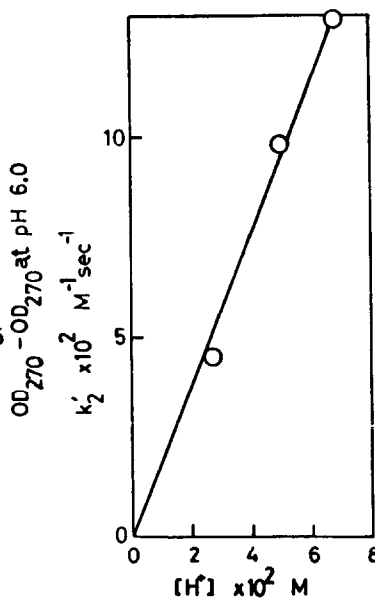


Fig. 2 Plots of  $k_2'$  versus  $[H^+]$ .

least-squares computation,  $k_0' \approx 0 (< 5 \times 10^{-4}) M^{-1} sec^{-1}$  and  $k_H' = 2.14 M^{-2} sec^{-1}$  resulted.

When medium pH was adjusted with additional buffer species other than HCl, they caused marked decrease in both the OD value and the  $k_2'$  value: for example,  $OD_{270} - OD_{270} (pH 6.0) = 0.100$  and  $k_2' = 0.036 M^{-1} sec^{-1}$  in the presence of 0.37 M of trichloroacetate buffer (pH 1.20). The result indicates that added buffer species catalyze the hydration equilibrium more efficiently than the reduction reaction.

In conclusion, the present study establishes that (1) the undissociated, free-ketone fraction of benzoylformic acid can be reduced in the nonenzymatic system, and (2) the reduction process is acid-catalyzed. Although the mode of the acid-catalysis (specific- or general-type) is not specified in this paper, general acid catalysis may be preferred on the basis of the detailed studies

in the related systems.<sup>6)</sup> In the enzymatic system, lactate dehydrogenase is active at neutral pH region(6-9)<sup>1)</sup>, indicating that pyruvate form( $\text{CH}_3\text{COCO}_2^-$ ) is employed as substrate and the hydration is almost dismissed.<sup>1,2,9)</sup> According to the X-ray crystallographic study<sup>1,2)</sup> pyruvate is bound to a cationic pocket constituted by protonated His 195 and Arg 171 in the style,  $\text{HisH}^+ \cdots \text{O}=\text{C}(\text{CH}_3)\text{CO}_2^- \cdots \text{H}^+\text{Arg}$ . It is most likely, on the basis of the present model investigation, that Arg 171 plays roles of not only binding the substrate but also neutralizing the anionic charge on the carboxylate function by forming the strong hydrogen-bond bridge, and  $\text{HisH}^+$  acts as acid source in the course of the reduction process.

#### References and Notes

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- (8)  $[\text{Bz1QH}]=0.019 \text{ M}$ ,  $[\text{C}_6\text{H}_5\text{COCO}_2\text{H}]=0.038 \text{ M}$ ,  $[\text{Cl}_3\text{CCO}_2\text{H}]=1.43 \text{ M}$ , and  $[\text{Cl}_3\text{CCO}_2\text{K}]=1.43 \text{ M}$  in a mixed solvent of water: isopropanol: tetrahydrofuran=20:5:5 in volume. Tetrahydrofuran was added to enhance the solubility of Bz1QH under the preparative reaction conditions. The reaction at 50°C gave mandelic acid in 6% yield after 6 h and 19% yield after 16 h.
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