Non-enzymatic Transamination and β-Elimination of DL-*S*-Benzylcysteine catalysed by a Potent Pyridoxal Model

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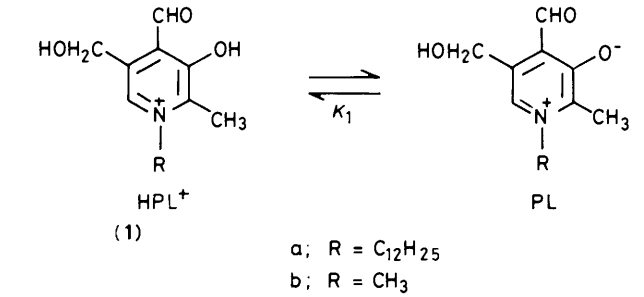
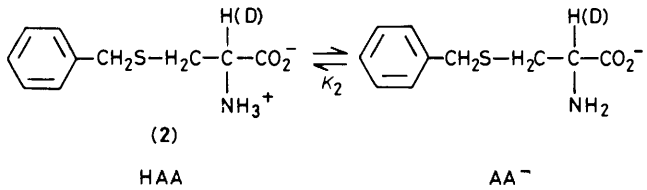
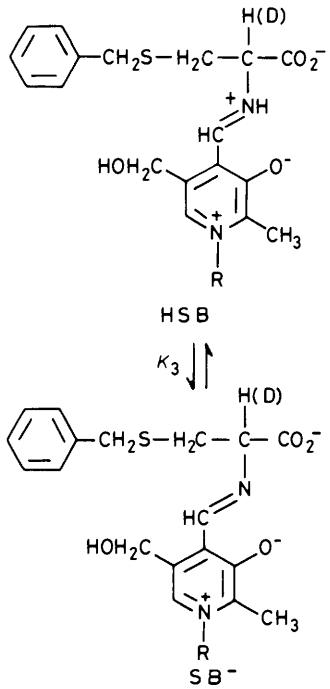
Non-enzymatic reactions of DL-*S*-benzylcysteine ($\mathbf{2}_H$) promoted by *N*-dodecylpyridoxal (**1a**) have been investigated in an aqueous hexadecyltrimethylammonium chloride (CTACl) micelle at 30.0 °C. The substrate undergoes mainly β-elimination at alkaline pH, whereas transamination with (**1a**) predominates in weakly acidic media. The kinetic α-deuterium isotope effects (k_H/k_D) of substrate were determined as 8.7 and 5.5 for β-elimination and transamination, respectively, at pH 7.0. These data indicate unambiguously that the rate-determining step of both reactions is the α-hydrogen abstraction from the reactive Schiff's base of substrate and (**1a**).

Pyridoxal 5′-phosphate (PLP) is a co-factor which plays an important role in the metabolic reactions of amino acids such as transamination and decarboxylation.[1] The function of PLP can be mimicked in part in model systems mostly in the presence of metal ions.[2] However, implications derived from such studies may be questioned as metal ions are not involved in the enzymatic reactions. Recently, we devised a pyridoxal model which does not rely on metal ions but can promote transamination of ordinary amino acids under mild conditions.[3] Our system consists of a pyridoxal carrying a dodecyl group on the pyridine nitrogen (**1a**) and hexadecyltrimethylammonium chloride (CTACl). In order to expand the scope of this potent model we have investigated catalysis of the reactions of DL-*S*-benzylcysteine by (**1a**) in a cationic micelle. This particular compound was chosen as a substrate, because the phenylmethanethiolate moiety is not only a hydrophobic but also a reasonably good leaving group. The former character is necessary for the favourable formation in the micelle of a Schiff's base of (**1a**) with substrate,[3] which is the key intermediate of the reaction. In this work, we attempted to elucidate the mechanism of the catalytic reaction of this amino acid by kinetic deuterium isotope effects.







Scheme 1.

Results

In conformity with previous observations,[3] hydrophobic *S*-benzylcysteine forms a Schiff's base with (**1a**) in 3mM-CTACl micelle with a large equilibrium constant *K* [equation (1) and

$$K = [SB^-]/[PL][AA^-] \quad (1)$$

Scheme 1]. The value determined by the procedures detailed in the Experimental section is as large as 31,000 l mol^{-1}. It is over 2,000-fold greater than the corresponding value for (**1b**) (15 l mol^{-1}). The favourable Schiff's base formation in the present system is due largely to the hydrophobic interaction of the apolar substituent of the substrate with the micelle. The notion that the Schiff's base (SB^-) resides in the less polar micellar phase is supported by a large red shift of its absorption from 380 nm for (**1b**) to 393 nm for (**1a**).[3] Since the pK_a of the Schiff's base of (**1a**) and substrate is unusually low (5.6), the Schiff's base exists largely in the ionized form (SB^-) in neutral to weakly alkaline media. The pH-distribution profile for (**1a**) and its Schiff's base is illustrated in Figure 1 for a solution of the composition identical with that of kinetic experiments.

It was found that there are two pathways for the reaction of the Schiff's base of (**1a**) and *S*-benzylcysteine (Scheme 2). One leads to β-elimination to give phenylmethanethiol, pyruvate, and ammonia. This reaction is the main pathway in the alkaline

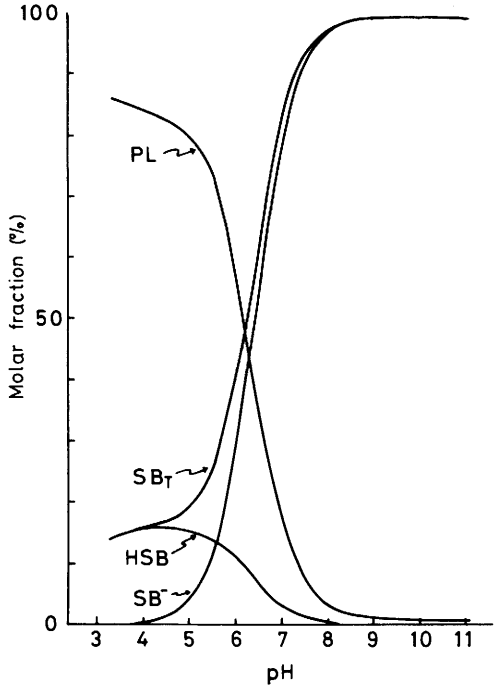


Figure 1. pH-Distribution profile for the Schiff's base of 0.25mM-(**1a**) and 5mM-($\mathbf{2_H}$) in 10mM aqueous buffer containing 3mM-CTACl

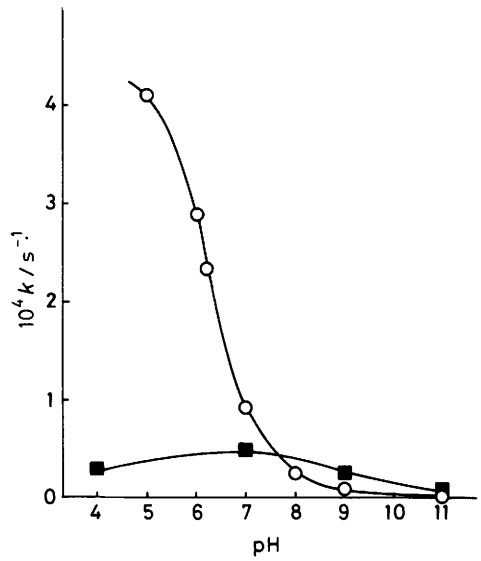
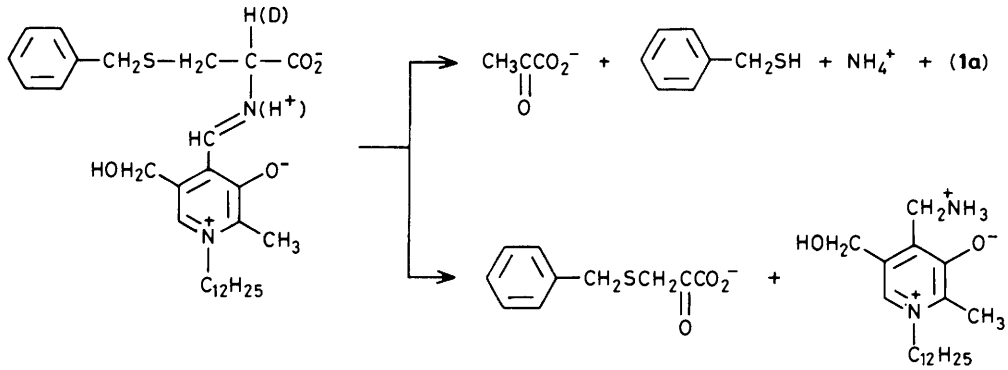


Figure 2. pH–Rate profiles for the transamination (○) and β-elimination (■) of the Schiff's base of (**1a**) and ($\mathbf{2_H}$) in 10mM aqueous buffer containing 3mM-CTACl and 1mM-EDTA at 30.0 °C



Scheme 2.

media, while transamination predominates in neutral to weakly acidic media (Figure 2). The pH–rate profile for the transamination reveals that the two ionic forms of the Schiff's base (HSB and SB^-) are distinctly different in reactivity. This observation is consistent with the results for the transamination of phenylalanine with (**1a**) under identical conditions,[4] and will not be described further.

Both transamination and β-elimination of *S*-benzylcysteine were found to be subject to a large kinetic deuterium isotope effect. A typical kinetic run for the elimination of undeuteriated ($\mathbf{2_H}$) and deuteriated substrate ($\mathbf{2_D}$) is shown in Figure 3. The specific rate constants for the two reactions at different pH and the isotope effects (k_H/k_D) derived thereof are compiled in the Table. An isotope effect as large as *ca.* 9 for the β-elimination of SB^- rules out the possibility that the α-hydrogen is abstracted at the pre-equilibrium stage, since if the α-proton abstraction were not involved in the rate-determining step no or little deuterium isotope effect would be observed. Rather, the observed large effect suggests that rate-determining α-proton abstraction is followed by fast departure of the phenylmethanethiolate group (Scheme 3). Alternatively, α-hydrogen abstraction and C–S bond scission may be taking place simultaneously in the transition state. In the transamination the isotope effect decreases with pH from 7.4 at pH 6.0 to 2.5 at pH 9.0, suggesting that the reaction mechanism changes over the pH range studied (see below).

Discussion

It was shown that (**1a**) promotes transamination and β-elimination of *S*-benzylcysteine in CTACl micelles under mild conditions. This efficient catalysis stems in large part from the favourable formation of the Schiff's base with substrate. The observed value of 31,000 l mol^{-1} for Schiff's base formation for *S*-benzylcysteine is the largest among the amino acids examined.[3] Presumably, a large proportion of the substrate carrying a hydrophobic substituent is partitioned in the apolar micellar phase in which (**1a**) resides, thereby enhancing Schiff's

Rate constants and kinetic deuterium isotope effects for the transamination and β-elimination of *S*-benzylcyteine (2_H) and (2_D) promoted by (**1a**) in 10mM aqueous buffer containing 3mM-CTACl and 1mM-EDTA at 30.0 °C

		(2_H)		(2_D)		
	pH	$10^5 k_{obs}^{H}/s^{-1}$	$10^5 k_H^a/s^{-1}$	$10^5 k_{obs}^{Db}/s^{-1}$	$10^5 k_D^a/s^{-1}$	k_H/k_D
Transamination	5.0	8.1	41.0			
	6.0	11.9	28.9	1.6	3.9	7.4
	6.2	11.5	23.4			
	7.0	7.7	9.3	1.4	1.7	5.5
	8.0	2.6	2.7	0.65	0.67	4.0
	9.0	0.84	0.84	0.33	0.33	2.5
	11.0	<0.01				
β-Elimination	4.0	0.47	2.9	~0.05	~0.3	
	7.0	4.0	4.8	0.46	0.55	8.7
	9.0	2.6	2.6	0.27	0.27	9.6
	11.0	0.53	0.53	~0.1	~0.1	

[a] Specific rate constants of the Schiff's base (k_H and k_D) were converted from k_{obs} by dividing the latter by the Schiff's base concentration at a given pH (*cf.* Figure 1). [b] The k_{obs}^{D} for (2_D) was obtained by correcting for the deuterium content of 92% in the sample used for the experiments. For details see Experimental section.

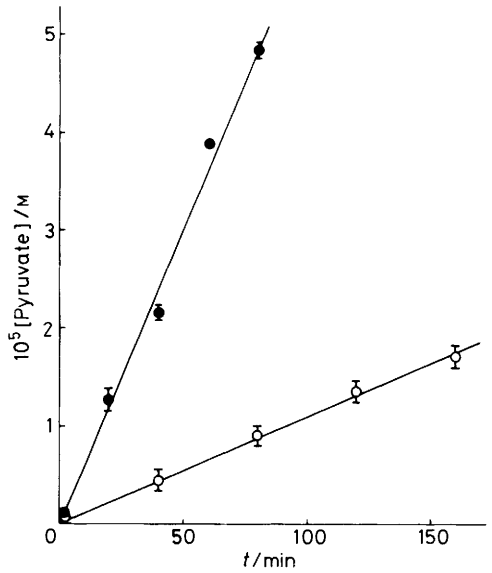
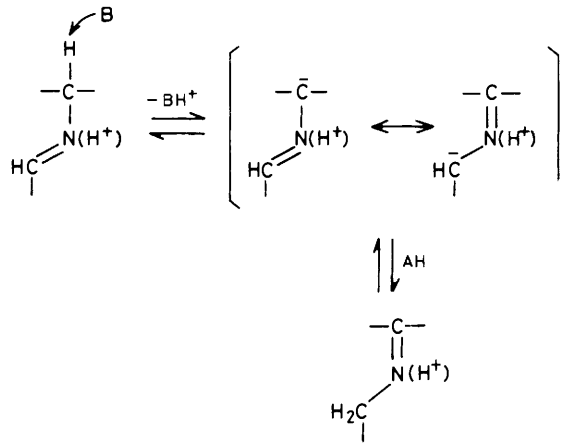


Figure 3. Kinetics of the β-elimination of 5mM-(2_H) (●) or (2_D) (○) promoted by 0.25mM-(**1a**) in 10mM-phosphate, pH 7.0 containing 3mM-CTACl and 1mM-EDTA at 30.0 °C. The bar refers to the scatter of duplicate runs



Scheme 3.

base formation.[4] Similar enhancements of the Schiff's base formation of PLP and related vitamins with amines and amino acids in micelles and artificial vesicles have been documented.[5–7] Another factor responsible for effective catalysis by (**1a**) is the presence of a positive charge on the pyridine nitrogen, which should labilise the α-hydrogen atom of the amino acid.[8,9] This aspect of catalysis is reminiscent of the enzymatic reactions mediated by aspartate aminotransferase, and probably by other PLP-dependent enzymes. According to a recent *X*-ray crystallographic analysis, the pyridine nitrogen of enzyme-bound PLP is protonated to form a tight hydrogen bond with an aspartate group of the enzyme active site.[10,11] Thus the pyridine ring carries a net positive charge during catalysis, thereby facilitating an electronic shift in the conjugated system.

It is often assumed that the PLP-catalysed reactions of amino acids proceed *via* a stable carbanion mechanism,[12,13] by which it is meant that fast α-hydrogen abstraction is followed by slow collapse of the resulting carbanion. This notion is based on the observation that many of the PLP-dependent enzymes readily exchange the α-hydrogen of amino acid with the solvent hydrogen.[14,15] Furthermore, a carbanionic intermediate that exhibits absorption at *ca.* 500 nm is observed in some (but not all) enzymatic and non-enzymatic reactions.[14,16] Nonetheless the relevance of these observations to actual reaction mechanisms remains somewhat equivocal. For one thing, kinetic deuterium isotope effects in non-enzymatic reactions do not appear to be compatible with the stable carbanion mechanism. For example, the β-elimination of *O*-phosphoserine in alkaline media is subject to an isotope effect (k_H/k_D) of 1.4—1.9.[17] The same reaction, when catalysed by glyoxalate and copper(II), exhibits an isotope effect of 1.1—3.4.[18] These data were interpreted as a β-elimination proceeding in a concerted manner by the *E*2 mechanism. Likewise the k_H/k_D value of 6—9 obtained for the transamination of alanine with pyridoxal analogues would be too large for the reaction proceeding *via* a stable carbanion.[8] The deuterium isotope effects observed for transamination and elimination in the present system are generally consistent with such data. In addition, the spectra of a transamination reaction mixture revealed no formation or accumulation of a stable carbanion intermediate.[3] Taken together, it is concluded that α-hydrogen abstraction is largely the rate-determining step in the non-enzymatic reactions. Hence, the overall reaction may be depicted by either one of two mechanisms. Slow α-hydrogen abstraction is followed by rapid

protonation at the azomethine carbon (transamination, Scheme 3) or by rapid departure of the C_β leaving group (elimination). Alternatively, these processes may take place completely in a concerted manner. If the former mechanism is valid for transamination, the observed decrease in the isotope effect with pH may be accounted for by the idea that protonation as well as α-hydrogen abstraction is rate determining.

In summary, these data suggest that the mechanism of non-enzymatic and enzymatic reactions may be different mainly in that α-hydrogen abstraction is rate determining in the former, while it is little or only partly so in the latter reaction. This difference could arise because in some enzyme reactions the chemical transformation of the substrate is so rapid that it is no longer a rate-determining step. In PLP-dependent enzymes such efficient catalysis appears to be conducted through general base catalysis of α-hydrogen abstraction by the lysine amino group that binds PLP in the absence of a substrate.[19,20] It was originally thought that hydroxide ions concentrated on the cationic micellar surface plays this role in the transformation of the Schiff's base,[21] but we are not certain whether this mechanism applies in our system. At any rate, incorporation of a 'powerful' base should improve the present catalyst to a true enzyme model at least with respect to the catalytic efficiency.

Experimental

Apparatus.—Electronic absorption spectra were determined on a Hitachi 200-10 or 220A spectrophotometer. Fluorescence spectra were taken on a Hitachi 650-10 fluorescence spectrophotometer. Mass spectra were recorded on a JEOL JMS-DX 300 mass spectrometer in the chemical ionization mode at 70 eV. pH Values of aqueous solutions were read on a Toa HM-5A pH meter fitted with a combination electrode GC-195C.

Materials.—Compounds (**1a** and **b**) were those used previously.[3] *S*-Benzyl-DL-cysteine ($\mathbf{2}_H$) and its α-deuteriated counterpart ($\mathbf{2}_D$) were prepared according to the literature.[22] The deuterium content in ($\mathbf{2}_D$) was determined as 92% by mass spectrometry;[23] ($\mathbf{2}_H$) (Found: C, 56.2; H, 6.2; N, 6.7. $C_{10}H_{13}NO_2S{\cdot}0.17H_2O$ requires C, 56.05; H, 6.3; N, 6.5%); ($\mathbf{2}_D$) (Found: C, 55.6; H, 6.25; N, 6.8. $C_{10}H_{12}DNO_2S{\cdot}0.25H_2O$ requires C, 55.5; H, 6.6; N, 6.5%). Hexadecyltrimethylammonium chloride (CTACl) was prepared by the method described earlier.[24] Rabbit muscle lactate dehydrogenase in an ammonium sulphate suspension (5 mg ml^{-1}) with a specific activity *ca.* 550 U mg^{-1} was obtained from Boehringer Mannheim. NADH and EDTA were purchased from Kyowa Hakko, Tokyo, and Dojindo, Kumamoto, respectively.

Schiff's Base Formation.—The pH-dependent equilibrium constant for the formation of Schiff's base of (**1a** or **b**) with ($\mathbf{2}_H$) or ($\mathbf{2}_D$) is defined by equation (2), where the suffix T stands for

$$K_{pH} = \frac{[\text{Schiff's base}]_T}{[(\mathbf{1a}) \text{ or } (\mathbf{1b})]_T \cdot [(\mathbf{2}_H) \text{ or } (\mathbf{2}_D)]_T} \quad (2)$$

sums of all ionic forms of individual components.[25,26] The corresponding pH-independent constant is defined by equation (1) for the least protonated species of each component. The pertinent equilibria are depicted in Scheme 1.

Aqueous solutions containing 0.25mM-(**1a** or **b**), varying concentrations (0—5 mM) of ($\mathbf{2}_H$) or ($\mathbf{2}_D$), and 1.0mM-EDTA in 10mM-buffer were prepared in the presence [(**1a**)] or absence [(**1b**)] of 3.0mM-CTACl. The buffer salts employed were the following: sodium acetate (pH 4.0 and 5.0), potassium dihydrogen phosphate (pH 6.0, 7.0, and 8.0), and sodium carbonate (pH 11.0). About 5 min after mixing when thermal and Schiff's base equilibria were attained, electronic absorption spectra were determined at 30.0 °C. The K_{pH} values at a given pH were obtained from a correlation of Schiff's base absorption with amino acid concentration. As the three components, pyridoxal, amino acid, and Schiff's base, exist solely in the least protonated form at pH 11, the K_{pH} determined at this pH is set equal to K. At low pH where HSB species is present in a significant amount, the Schiff's base concentration was estimated at an isosbestic point of HSB and SB^- [402 nm for the Schiff base of (**1a**)]. From the known extinction coefficient of SB^- (and of HSB) and the observed absorbance there, the total Schiff's base concentration is evaluated. The K_{pH} values thus determined at different pH are shown in Figure 4. These data were used to optimize the unknown parameters pK_{a2} and pK_{a3} for acid dissociation constants of *S*-benzylcysteine and Schiff's base, respectively, in the CTACl micelle. Their respective values of 8.5 and 5.6 along with pK_{a1} 2.6 and K 31,000 l mol^{-1} determined separately were used to draw the theoretical curve. Neither K nor pK_{a3} appears to be affected significantly by deuterium substitution on the substrate, because the Schiff's base absorption soon after mixing with (**1a**) is identical within experimental error for the two compounds ($\mathbf{2}_H$) and ($\mathbf{2}_D$) at all pH values studied, 5.0, 6.0, 7.0, 8.0, 9.0, and 11.0.

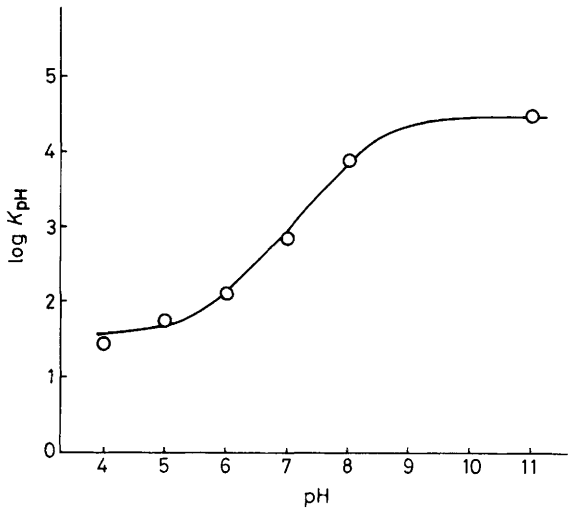


Figure 4. Correlation of log K_{pH} for the formation of a Schiff's base between (**1a**) and ($\mathbf{2}_H$) with pH at 30.0 °C. Open circles represent experimental data and the theoretical curve was drawn using the following parameters: pK_{a1} 2.6, pK_{a2} 8.5, pK_{a3} 5.6, and K 31,000 l mol^{-1}

Kinetics.—A reaction mixture (3 ml) containing 5.0mM-($\mathbf{2}_H$) or ($\mathbf{2}_D$), 1.0mM (EDTA, and 3.0mM-CTACl in 10mM-buffer was placed in a cuvette at 30.0 °C. To this was added 42.5 μl of a stock solution of (**1a**), the concentration of (**1a**) being kept at 0.25mM. Transamination was followed by a decrease in the absorption of the Schiff's base at its $\lambda_{max.}$. Kinetic data were analysed in a manner analogous to those described previously.[3] All experiments were carried out in duplicate and the rate constants presented are reproducible to within ±7% of the mean. Since the deuterium content in ($\mathbf{2}_D$) is 92%, the rate constant determined with this sample (k_{obs}') is converted into k_{obs}^D by using relationship (3)

$$k_{obs}^D = (k_{obs}' - 0.08\, k_{obs}^H)/0.92 \quad (3)$$

The β-elimination of substrate was carried out at 30.0 °C in a reaction solution of the same composition as above. Portions were withdrawn at time intervals and a portion (0.10 ml) was

transferred to a fluorescence cuvette containing 2.9 ml of 0.10M-phosphate buffer (pH 7.0) at 30.0 °C. After 5 min an NADH solution (4 μl) in 0.10M-carbonate buffer (pH 10.6) was injected to bring the NADH concentration to *ca.* 1.5×10^{-6}M. After a further 3 min this was followed by addition of a suspension of lactate dehydrogenase (1 μl). A decrease in fluorescence intensity of NADH, which results from the enzymatic reduction of pyruvate to lactate, was followed at 460 nm with excitation at 340 nm. In a control experiment where substrate was omitted no decrease in the fluorescence of NADH was observed. Reaction rates (v) were linear with time in the initial stage of the reaction (Figure 3). Combination of a rate equation [equation (4)] and K_{pH} [equation (2)] yields equation (5), from which the specific rate constant of the Schiff's base (k) is obtained.

$$v = k[SB]_T \quad (4)$$

$$v = kK_{pH}[AA]_0[PL]_0/(1 + K_{pH}[AA]_0) \quad (5)$$

Acknowledgements

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