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Studies on the Binding Mechanism of VB_1 and VB_9 with Trypsin

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ABSTRACT

The binding characteristics of vitamin B_1 (VB_1) and vitamin B_9 (VB_9) with trypsin were investigated by fluorescence spectrometry and UV/vis spectrophotometry under simulated physiological conditions. With the addition of VB_1 or VB_9, the intrinsic fluorescence emission intensity of trypsin was quenched by the nonradiative energy transfer mechanism. The fluorescence quenching process of trypsin may be mainly governed by a static quenching mechanism. The binding parameters such as the binding constants and the number of binding sites can be evaluated by fluorescence quenching experiments. The numbers of the apparent binding constant K_b of VB_1-trypsin at different temperatures were 0.4948 and 4.8340×10^4 L/mol and the numbers of binding sites n were 0.9359 and 1.1820. Similarly, the numbers of the apparent binding constant K_b of VB_9-trypsin at different temperatures were 5.9310 and 13.040×10^4 L/mol and the numbers of binding sites n were 0.9908 and 1.0750. The thermodynamic parameters, with a negative value of ΔG, revealed that the bindings are spontaneous processes and the positive values for both enthalpy change (ΔH) and entropy change (ΔS) indicate that the binding powers of VB_1 and VB_9 with trypsin are mainly hydrophobic interactions. And synchronous spectrums were used to study the conformational change of trypsin. In addition, the binding distances of VB_1-trypsin and VB_9-trypsin were estimated to be 0.55 nm and 0.87 nm according to the Förster's resonance energy transfer theory.

Keywords: Trypsin; VB_1 and VB_9; Fluorescence Spectrometry; Nonradiative Energy Transfer Mechanism

1. Introduction

There are main functions of VB_1 in sugar metabolism, energy metabolism and digestive system normal work [1]. VB_9 is the floorboard of the compounds which contains pteroylglutamic acid and is well studied and separated from spinach leaf [2]. Proteins are important and widespread in kinds of biological macromolecules in living organisms and take part in almost all life processes. Trypsin is a serine proteinase, which hydrolyzes proteins and peptides at the carboxyl sides of arginine and lysine residues. Many investigations between proteins and vitamins have been published including bovine serum albumin [3-6] and human serum albumin [7-9], but the studies on the interaction between vitamin B and trypsin have not been reported. In this paper, the interactions between vitamin B and trypsin have been studied at different temperatures under physiological conditions using UV/vis spectrophotometry and fluorescence spectrometry. The effects of VB_1 and VB_9 on the trypsin have been evaluated and compared, such as quenching mechanism, binding constants, binding sites, binding mode and so on.

2. Experimental

2.1. Apparatus and Reagents

An FP-8300 fluorescence spectrometer (Jasco, Japan) was used to record the fluorescence spectra in 1.00 cm quartz cell, a TU1901 UV/vis Spectrophotometer (PGeneral, Beijing, China) was employed to record the absorption spectra and a PHS-3C meter (Shanghai Precision Scientific Instrument Co., Ltd China) was used to measure the pH values of B-R buffer solutions.

VB_1 solutions (1.00×10^{-4} mol/L) were prepared by diluting 0.0094 g (337.27 Da, Sinopharm Chemical Rengent Co., Ltd, Shanghai, China) in 250.00 mL of deionized water. VB_9 solutions (1.00×10^{-4} mol/L) were dissolved by diluting 0.0110 g (441.41 Da, Tianjin recovery fine chemical industry research institute, Tianjing, China) in 250.00 mL of deionized water. Trypsin solutions (1.00×10^{-4} mol/L) were prepared by diluting 0.6005 g of trypsin (24000 Da, Sinopharm Chemical Rengent Co.,

Ltd, Shanghai, China) in 250.00 mL of water. Britton-Robinson (B-R) buffer solutions (pH = 7.90) were prepared by combining a mixed acid (composed of 0.04 mol/L of H_3PO_4, HAc, and H_3BO_3) with 0.20 mol/L of NaOH in equal proportions. NaCl (0.20 mol/L) were dissolved to adjust the ionic strength of the VB_1-trypsin and VB_9-trypsin solutions so as to study the effects of electrolytes on binding. All solutions were prepared using double-distilled, deionized water and the reagents were of analytical reagent grade. In the experiments, a known volume standard of VB_1 or VB_9 solutions were added in 10.00 mL calibrated tubes with deionized water and mixed well.

2.2. General Procedure

In 10.00 mL calibrated tubes, 1.00 mL B-R buffer solutions (pH = 7.90), 1.00 mL of 1.00×10^{-4} mol/L trypsin solutions and a known volume of the standard VB_1 or VB_9 solutions were added. Then the mixture were diluted to 10.00 mL with NaCl (0.20 mol/L) and mixed thoroughly by shaking. After reaction for 30 min, the solutions were taken into the optical cell. The system's fluorescence spectra wavelengths were recorded from 290 nm to 450 nm and the bandwidths were 5 nm.

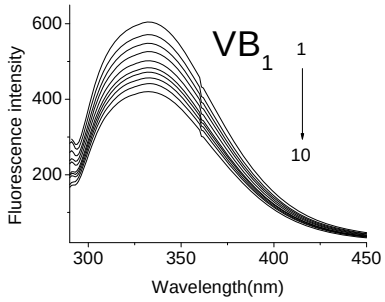
3. Results and Discussion

3.1. Fluorescence Quenching Spectra and Quenching Mechanism of VB_1 and VB_9 with Trypsin

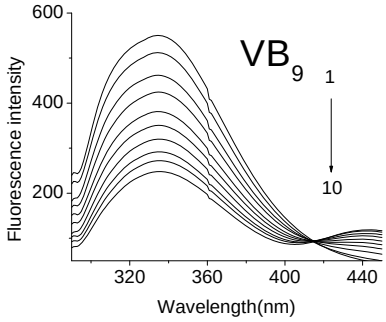
Figure 1 shows that fluorescence emission spectra of trypsin with the increasing concentrations of VB_1 and VB_9 following an excitation wavelength at 281 nm. Trypsin shows a fluorescence emission with a peak at 340 nm. The fluorescence intensity of trypsin decreased gradually with the increasing concentrations of vitamin B, and higher concentrations led to more efficient quenching of the tryptic fluorescence. By comparison, it was known that VB_9 led to more apparently efficient quenching of the protein fluorescence than VB_1. Such a quenching clearly indicated the binding of VB_1 and VB_9 with trypsin. Meanwhile, there are not a shift of maximum emission peaks, indicating that vitamin B didn't influence the microenvironment around trypsin. The fluorescence quenching mechanisms usually contain dynamic quenching and static quenching, which are caused by diffusion and ground-state complex formation spectively [10,11]. In order to further clarify the fluorescence quenching mechanism induced by vitamin B, the Stern-Volmer equation get used to evaluate the data.

$$\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + K_q \tau_0 [Q] \quad (1)$$

where F_0 and F represent the steady-state fluorescence intensities in the absence and presence of the quencher,



(a)



(b)

Figure 1. (a) Evolution of fluorescence spectra of trypsin in presence of VB_1 with different concentrations. (b) Evolution of fluorescence spectra of trypsin in presence of VB_9 with different concentrations. [Trypsin] = 1.0×10^{-5} mol/L; [VB_1] × 10^{-5} mol/L, 1-10: 0.00, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, [VB_9] × 10^{-5} mol/L, 1-10: 0.00, 0.10, 0.30, 0.50, 0.70, 0.90, 1.10, 1.30, 1.50, 1.70, λ_{ex} = 281 nm, λ_{em} = 340 nm; pH = 7.90; T = 300 K.

respectively; $[Q]$ is the concentration of quencher; K_{sv} is the Stern-Volmer quenching constant; K_q is the bimolecular quenching rate constant and K_q is equal to K_{sv}/τ_0; τ_0 is the average lifetime of the molecule without any quencher and this τ_0 = 10^{-8} s [12]. The Stern-Volmer curves at two temperatures were shown in **Figure 2**. The values of K_{sv} and K_q derived from Equation (1) are listed in **Table 1**. The minimum value of K_q as shown in **Table 1** is 8.621×10^{11} L/(mol·s), which is greater than the maximum diffusion collision quenching rate constant of 2.0×10^{10} L/(mol·s) [13]. So it indicated that the fluorescence quenching process of trypsin with VB_1 and VB_9 may be mainly governed by a static quenching mechanism.

3.2. Binding Constant and Number of Binding Site

In static quenching process, when small molecules are bound independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by Equation (2) [14]:

$$\log \frac{F_0 - F}{F} = \log K_b + n \log[Q] \quad (2)$$

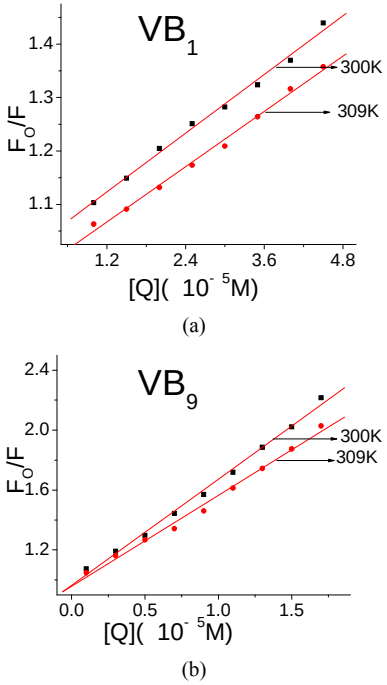


Figure. 2. The Stern-Volmer plots of the trypsin-VB_1 and trypsin-VB_9 systems at different temperatures.

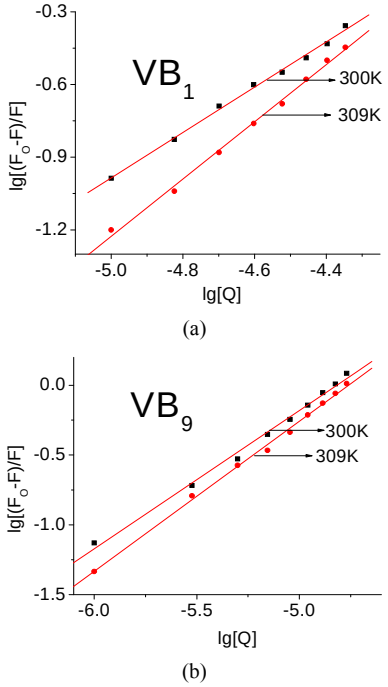


Figure 3. lg(F_0-F)/F against lg[Q] at two temperatures of two systems.

Table 1. Stern-Volmer quenching constants for the VB_1-trypsin and VB_9-trypsin systems at pH = 7.9.

Drug	T K	K_{SV} 10^4 L/mol	R	K_q 10^{12}L/(mol·s)
VB_1	300 ± 1	0.9159	0.9977	0.9159
	309 ± 1	0.8621	0.9977	0.8621
VB_9	300 ± 1	7.0920	0.9973	7.0920
	309 ± 1	6.0720	0.9963	6.0720

Table 2. Binding constants and thermodynamic parameters of VB_1 and VB_9 with trypsin at two temperatures.

Drug	T K	K 10^5 L/mol	n	R	ΔG KJ/mol	ΔH KJ/mol	ΔS J/(mol·K)
VB_1	300	0.4948	0.9359	0.9982	−21.22	195.2	721.3
	309	4.8340	1.1820	0.9981	−21.71	195.2	721.3
VB_9	300	5.9310	0.9908	0.9971	−27.41	67.46	316.2
	309	13.040	1.0750	0.9986	−30.26	67.46	316.2

where K_b is the binding constant and n is the number of binding sites. The values for K_b and n at different temperatures can be derived in **Figure 3** from the intercept and slope of plots of $\log\frac{F_0 - F}{F}$ versus $\log[Q]$ based on Equation (2), which are listed in **Table 2**. K_b shows that there are a strong interaction and a complex formation between trypsin with VB_1 and VB_9. Furthermore, it can be inferred from the values of n that there is an independent class of binding sites on trypsin with VB_1 and VB_9. But it appears that the binding constants and the number of binding sites also increase with higher temperature [15,16]. So this may be because the capacity of VB_1 and VB_9 binding to trypsin is enhanced with increasing temperature.

3.3. Thermodynamic Parameters and Nature of Binding Mode

The intermolecular forces contributing to drug-biomolecule interactions with drugs may include hydrogen bonds, Van der Waals interactions, electrostatic interactions and hydrophobic force, etc. The thermodynamic parameters were analyzed by temperature in order to provide strong evidence for the presence of binding forces. The value of enthalpy change (ΔH) and entropy change (ΔS) can be determined by the van't Hoff Equation (3), if the enthalpy change (ΔH) does not vary significantly with temperature. The value of free energy change (ΔG) for a binding interaction at different temperatures can be determined by the Equation (4).

$$\ln K_b = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

$$\Delta G = -RT \ln K_b \quad (4)$$

where K_b is the binding constant, R is the gas constant and T is the absolute temperature. The values of ΔH, ΔS and ΔG are listed in **Table 2**. A negative value of ΔG reveals that the binding process is spontaneous. Hydrophobic interactions play main roles in the binding between trypsin with VB_1 and VB_9 because the values of ΔH and ΔS are positive.

3.4. Synchronous Fluorescence Spectroscopy

Synchronous fluorescence spectroscopy is usually used to investigate the microenvironment around the fluorophore functional groups. At $\Delta\lambda$ = 60 nm, the synchronous fluorescence spectra are attributed to tryptophan, while $\Delta\lambda$ = 15 nm, the spectra are attributed to tyrosine. Synchronous fluorescence spectra of trypsin with addition of VB_1 are shown in **Figure 4(a)** and these with addition of VB_9 are shown in **Figure 4(b)**. From **Figure 4**, the emission maxima have no shifts with regards to VB_1 and VB_9, which indicates that there was no change of the microenvironment of the tryptophan and tyrosine.

3.5. Energy Transfer and Binding Distance

The fluorescence quenching of trypsin after binding with VB_1 and VB_9 indiactes that the tranfer of energy has occurred. According to Förster's resonance energy transfer theory [17], the distance between two interacting molecules and the efficiency of energy transfer can be discribed by the following equation:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + R^6} \tag{5}$$

where E is the energy transfer efficiency, F is the fluorscence intensity of the donor in the presence of equal amounts of accepter, F_0 is the fluorscence intensity of the donor in the absence of equal amounts of accepter, R_0 is the critical distance, and R is the distance between acceptor and donor. The quantity R_0^6 is calculated by the following equation:

$$R_0^6 = 8.8\times10^{-25} K^2 N^{-4} \Phi J \tag{6}$$

where K^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor, J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. J is calculated using the equation:

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{7}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescence donor when the wavelength is λ and $\varepsilon(\lambda)$ is the molar absorbance coefficient of the acceptor when the wavelength is λ. It has been reported that K^2 = 2/3, N = 1.336, and Φ = 0.118. **Figure 5** shows that the spectral overlap between the fluorescence emission spectrum of trypsin and UV/vis absorption spectrum of VB_1 and VB_9. From the above relationships, $J = 3.79\times 10^{-19}$ $cm^3\cdot l\cdot mol^{-1}$, R_0 = 0.45 nm, E = 0.79 and R = 0.55 nm for trypsin and VB_1. Similarly, $J = 7.96 \times 10^{-17}$ $cm^3\cdot l\cdot mol^{-1}$, R_0 = 0.87 nm, E = 0.23 and R = 0.87 nm for trypsin and VB_9. The distance R < 8 nm between donor and acceptor indicates that the energy transfer from trypsin to VB_1 and VB_9 occurred with high possibility. This obeyed the conditions of Förster energy transfer theory.

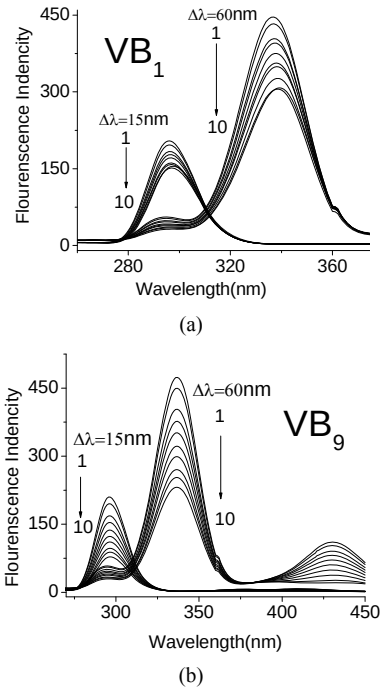


Figure 4. (a) Evolution of synchronous fluorescence spectra of trypsin in the absence and presence of VB_1; (b) Evolution of synchronous fluorescence spectra of trypsin in the absence and presence of VB_9, T = 300 K.

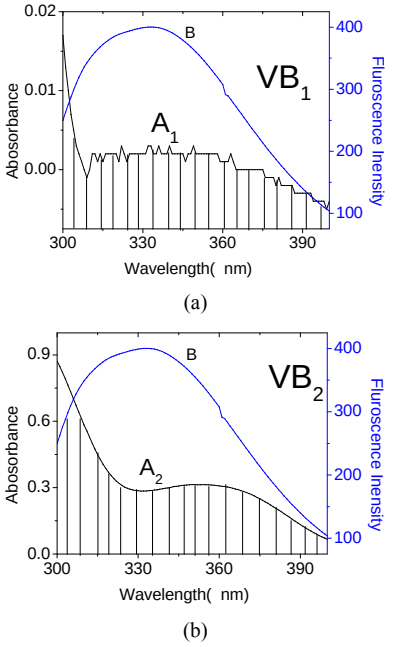


Figure 5. Spectral overlap between fluorescence spectrum of trypsin (B) and absorbance spectrum of VB_1 (A_1) and VB_9 (A_2) at 300 K; [trypsin] = [VB_1] = 5.0 × 10^{-5} mol/L, [trypsin] = [VB_9] = 5.0 × 10^{-5} mol/L.

4. Conclusion

In this paper, the interactions between two kinds of vitamin B and trypsin have been investigated under simulated physiological conditions using spectrometries. The fluorescence of trypsin was quenched by two kinds of vitamin B mainly through static quenching. The enthalpy change (ΔH) and entropy change (ΔS) for the systems were calculated respectively. The positive ΔH and ΔS values indicated that hydrophobic interactions played main roles in the binding between trypsin and vitamin B. A binding distance R of 0.55 nm and 0.87 nm between donor and acceptor was obtained. According to the data, the two B vitamins have similar interactions with trypsin. The results obtained are of important biological significance in pharmacology and clinical medicine.

5. Acknowledgements

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REFERENCES

[1] H. B. Xue, C. Y. Li, L. C. Gao and G. Q. Zhang, "The Interaction of VB_1 and Bovine Serum Albumin," *Chinese Journal of Analysis Laboratory*, Vol. 30, No. 4, 2011, pp. 111-114.

[2] H. J. Liu, P. Li, Y. D. Zhang, C. Guo, J. Y. Deng, J. W. Cai and B. L. Liu, "Spectroscopic Study on Binding of Folic Acid to Human Serum Albumin," *Spectroscopy and Spectral Analysis*, Vol. 29, No. 7, 2009, pp. 1915-1919.

[3] Y. H. Shang, H. Li, J. J. Sun and M. Y. Zhang, "Study on the Interaction of Bovine Serum Albumin with Riboflavin by Fluorescence Spectroscopy," *Journal of Anal Ytical Science*, Vol. 26, No. 1, 2010, pp. 67-70.

[4] L. Q. Sheng, X. Y. Yan, H. J. Xu, H. W. Tong and S. M. Liu, "Study on the Interaction between BSA and Nicotine," *Spectroscopy and Spectral Analysis*, Vol. 27, No. 2, 2007, pp. 306-308.

[5] A. M. Zhang, "Study on the Interaction Between VB_2 and Serum Albumin by Fluorescence Spectroscopy," *Journal of Nanchang University*, Vol. 30, Suppl., 2006, pp. 1087-1088.

[6] Y. H. Fan, F. Feng, Z. Z. Chen and S. M. Shuang, "Studies on Interaction Between Bovine Serum Albumin and Vitamin B_{12} by Fluorescence Spectrometry," *Chinese Journal of Spectroscopy Laboratory*, Vol. 28, No. 3, 2011, pp. 1331-1335.

[7] H. J. Liu, P. Li, Y. D. Zhang, C. Guo, J. Y. Deng, J. W. Cai and B. L. Liu, "Spectroscopic Study on Binding of Folic Acid to Human Serum Albumin," *Chinese Journal of Spectroscopy Laboratory*, Vol. 29, No. 7, 2009, pp. 1915-1919.

[8] J. L. Wang, L. C. Fu, S. W. Zhou, Z. J. Chen, W. B. Lu, X. M. Ye, G. Z. Meng and S. Z. Fu, "The Interaction of Vitamin B_6 with the Human Serum Albumin," *Spectroscopy and Spectral Anaiysis*, Vol. 25, No. 6, 2005, pp. 912-915.

[9] N. Zhang, C. X. Xu, Q. Wei, B. Du, R. Li, T. G. Zhang, D. Wu and Y. X. Dai, "Studies on the Interaction of DNA with Vitamin B_{12} Based on the Immobilization of dsDNA on Nano-Scale Hydroxyapatitle Coating," *Advanced Materials Letters*, Vol. 1, No. 1, 2010, pp. 34-39. http://dx.doi.org/10.5185/amlett.2010.3104

[10] Z. Q. Jing, Y. H. Chi, J. Zhuang, X. Y. Bi and L. Zhou, "Mechanism Studies on the Combination Reaction between Bovine Serum Albumin and Zincon by Fluorescence Spectra," *Spectroscopy and Spectral Analysis*, Vol. 27, No. 5, 2007, pp. 986-990.

[11] J. H. Tang, S. D. Qi and X. G. Chen, "Spectroscopic Studies of the Interaction of Anticoagulant Rodennticide Diphacinone with Human Serum Albumin," *Journal of Molecular Structure*, Vol. 779, No. 1-3, 2005, pp. 87-95. http://dx.doi.org/10.1016/j.molstruc.2005.07.023

[12] J. R. Lakowicz and G. Weber, "Quenching of Fluorescence by Oxygen, Probe for Structural Fluctuations in Macromolecules," *Biochemistry*, Vol. 12, No. 21, 1973, pp. 4161-4170. http://dx.doi.org/10.1021/bi00745a020

[13] X. Z. Feng, Z. Lin, L. J. Yang, C. Wang and C. L. Bai, "Investigation of the Interaction between Acridine Orange and Bovine Serum Albumin," *Talanta*, Vol. 47, No. 5, 1998, pp. 1223-1229. http://dx.doi.org/10.1016/S0039-9140(98)00198-2

[14] M. Jiang, M. X. Xie, Y. Liu, X. Y. Li and X. Chen, "Spectroscopic Studies on the Interaction of Cinnamic Acid and Its Hydroxyl Derivatives with Human Serum Albumin," *Journal of Molecular Structure*, Vol. 692, No. 1-3, 2004, pp. 71-80. http://dx.doi.org/10.1016/j.molstruc.2004.01.003

[15] T. Förster, "Modern Quantum Chemistry," In: O. Sinaoglu, Ed., Vol. 3, Academic Press, New York, 1965.

[16] G. Cristobal, R. Dos and D. M. Pierre, "Fluorescence Resonance Energy Transfer Spectroscopy Is a Reliable 'Ruler' for Measuring Structural Changes in Proteins: Dispelling the Problem of the Unknown Orientation Factor," *Journal of Structural Biology*, Vol. 115, No. 2, 1995, pp. 175-185. http://dx.doi.org/10.1006/jsbi.1995.1042

[17] D. C. Saha, K. Ray and T. N. Misra, "Energy Transfer in Triton-X 100 Micelles: A Fluorescence Study," *Spectrochimica Acta Part A*, Vol. 56, No. 4, 2000, pp. 797-801. http://dx.doi.org/10.1016/S1386-1425(99)00169-9