# Binding Set Analysis for Interaction of Human Serum Albumin with Cethyl Trimethylammonium Bromide

**Abdol-Khalegh Bordbar,* Nasrin Sohrabi, and Hossain Gharibi†**

*Department of Chemistry, Isfahan University, Isfahan 81746-73441, Iran*
*†Department of Chemistry, Tarbiat-Modares University, Tehran, Iran*
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The binding of cethyl trimethylammonium bromide, (CTAB) with human serum albumin (HSA) has been investigated at 5 mM phosphate buffer pH 7.0, 27 °C and various ionic strength using ion selective membrane electrodes. This method is faster and much more accurate than equilibrium dialysis technique, so provides sufficient and accurate data for binding data analysis. A novel and simple method was introduced for resolution and characterization of binding sets on basis of binding capacity concept. The values of Hill binding parameters were estimated for each set and used for calculation of intrinsic binding affinity. The results interpreted on basis of nature of forces which interfered in the interaction and represent the existence of three and two binding sets for binding of CTAB at $10^{-4}$ and $10^{-3}$ M of NaBr, respectively.

**Key Words :** Binding capacity, Scatchard plot, Ionic surfactant, Human serum albumin

## Introduction

Human serum albumin (HSA) is the highly water-soluble plasma protein which is the smallest and most abundant plasma protein in the human body, accounting for 55% of the total protein in blood plasma. HSA is a single-stranded polypeptide whose amino acid sequence is known.[1] Its ionizable groups include 116 total acidic groups (98 carboxyl and 18 phenolic-OH) and 100 total basic groups(60 amino, 16 imidazolyl, 24 guanidyl). The absolute molecular weight of 66436 D was calculated from the numbers and molar masses of the consistent amino acid residues, which yields a contour length of the denatured protein of L contour(HSA)=216 nm(as calculated from the number of residues and a peptide bond length of 0.37 nm). HSA contains 17 disulfide bridges, one free thiol(cys 34) and a single tryptophan typically bind 1-2 fatty acids per protein,[2] which effectively reduces the isoelectric point in 0.15 M NaCl, the pI for lipid-bound HSA is 4.7,[3] while pI=5.7 for defatted HSA.[4] At pH 7.4, the shape of native HSA in solution is thought to be a prolate ellipsoid of revolution with major and minor axes, respectively, 12.0 and 2.7 nm[5] or 14.1 and 4.1 nm,[6,7] linking three homologous, globular domains in series.

Interaction between ionic surfactants and globular proteins has been extensively studied as the ligand binding affinities.[8,9] It is important for understanding the stabilization of membranes, food emulsions and foams that the interactions between the protein and surfactant which leads to the formation of such complexes are characterized.[10] Surfactants can be broadly divided into those which bind and initiate protein unfolding, *i.e*. denaturating surfactants, and those that only bind leaving the tertiary structure of the protein intact. Commonly used anionic surfactants, such as sodium n-dodecyl sulfate (SDS), generally denature proteins where as non-ionic surfactants do not.[11,12]

The binding data for ionic surfactant-protein interaction can be measure experimentally, using equilibrium dialysis[13] and potentiometric techniques.[14] Binding data analysis can reveal some important features of binding mechanism. However, different models of analysis that depend on various features of the binding process are required for this purpose. A number of methods for graphical and computer-assisted analysis of the binding data for surfactant-protein interaction have been employed.[13,15,16] One of the most common presentations of such data is the Scatchard plot.[17] However, there is not a general and comprehensive method for characterization of binding sets in surfactant-protein system.

In the present study, the interaction of cethyl trimethyl-ammonium bromide (CTAB) with HSA has been studied using ion selective membrane electrode as a fast and accurate technique. A novel graphical method has been introduced for binding data treatment and the results have been interpreted on basis of binding mechanism and leads to the formation of CTAB-HSA complexes are characterized.

## Materials and Method

HSA (free fatty acid fraction V) and CTAB were purchased from Sigma Chemical Co., Tetrahydrofurane (THF), acetone, nitric acid (65%), sodium hydroxide, sodium bromide, panta oxide diphosphor ($P_2O_5$), ethanol, carboxylated PVC, high molecular weight, sodium phosphate and sodium hydrogen phosphate were obtained from Merck Chemical Co. Silver wire and sodium reference electrode (serial num. 6.0501. 100) were purchased from Metrohm Co. All the materials have high degree of purity. All of the solutions were prepared by double distilled water. The 5 mM phosphate

*Corresponding Author. Tel: +98-311-7932710; Fax: +98-311-6689732; e-mail: bordbar@sci.ui.ac.ir

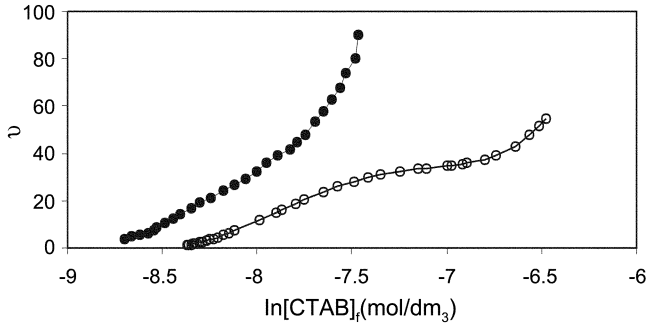


**Figure 1**. The binding isotherms for interaction of CTAB with HSA at pH=7.0, 5 mM phosphate buffer and 27 °C. (●) $10^{-3}$ M and (○) $10^{-4}$ M of NaBr.

buffer pH 7.0 was used as buffer. The HSA solutions were freshly prepared and used. For all the potentiometric measurements we used potentiometer of Metrohm model, 744.

**Potentiometry**. Free CTAB concentration was determined by means of a CTAB-selective plastic membrane electrode which has been reported to have an excellent CTAB selectivity and a Nernstian response.[14] The reference electrode was sodium electrode.

**Binding data analysis and results**. Figure 1 is the binding isotherms for interaction of CTAB with HSA and shows the variation of $\nu$ (the average number of bound CTAB per HSA molecule)versus $\ln[CTAB]_f$. The corresponding Scatchard plots for these isotherms are shown in Figure 2. These are not coincidence with usual shapes of Scatchard plots and can be represent the existence of more than one binding set. However, the resolution and characterization of binding sets is difficult through these plots. For overcoming to this deficiency, one can use the concept of binding capacity. Binding capacity is the homotropic second derivative of the binding potential with respect to the chemical potential of the ligand ($\mu_S$) and provides a measure of steepness of the binding isotherm.[18] It represents the changes in the number of mole of ligand per mole of the macromolecule ($\nu$) that accompanies a change in the chemical potential of that ligand. The heat capacity and the compressibility define analogous concepts with respect to temperature and pressure, respectively. By considering the

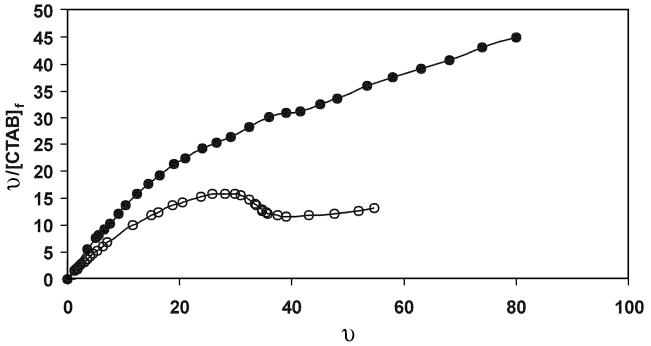


**Figure 2**. The Scatchard plots for interaction of CTAB with HSA at pH=7.0, 5 mM phosphate buffer and 27 °C. (●) $10^{-3}$ M and (○) $10^{-4}$ M of NaBr.

ideal behavior ($\mu_s = \mu_s^0 + RT \ln[S]_f$), binding capacity ($\theta$) equals to:

$$\theta = \left(\frac{\partial \upsilon}{\partial \mu_s}\right)_{T,P,\mu_{j\neq s}} = \left(\frac{\partial \upsilon}{RT\partial \ln[S]_f}\right)_{T,P,\mu_{j\neq s}} \quad (1)$$

Where $[S]_f$ is the free concentration of the ligand. This concept is directly related to the type and the extent of cooperativity.[18] This parameter can be measured directly by using experimental techniques for some systems such as binding of oxygen to hemoglobin.[19] However, using computer program for fitting of the binding isotherms in an appropriate equation and then calculating the slope of binding isotherm through fitting equation, the values of $\theta$ at any $\nu$ can be determined.

It can be written for system with N-independent binding sets:

$$\upsilon = \sum_{i=1}^{N} \upsilon_i \quad \text{and} \quad \left(\frac{\partial \upsilon}{\partial \upsilon_i}\right) = 1 \quad (2)$$

Where $\nu_i$ is the average number of bound ligand in the ith binding set per macromolecule. With respect to equation (1), $\theta$ of this system is as follows :

$$\theta = \frac{\partial \upsilon}{RT\partial \ln[S]_f} = \sum_{i=1}^{N} \left(\frac{\partial \upsilon_i}{RT\partial \ln[S]_f}\right) = \sum_{i=1}^{N} \theta_i \quad (3)$$

Where $\theta_i$ is the contribution of the ith binding set in overall binding capacity. The Hill equation of this system is[20,21]

$$\upsilon = \sum_{i=1}^{N} \frac{g_i (K_i[S]_f)^{n_{Hi}}}{(1 + (K_i[S]_f)^{n_{Hi}})} \quad (4)$$

Where $g_i$, $K_i$ and $n_{Hi}$ are the number of binding sites, binding constant and Hill coefficient for ith binding set, respectively. Using equations (1) and (4), it can be written :

$$\theta_i = \frac{n_{Hi}\upsilon_i(g_i - \upsilon_i)}{g_i RT} \quad (5)$$

With respect to equation (3) and (5), the binding capacity curve should be the summation of N-Gaussian curve that each of them relates to the corresponding binding set. If $K_i >> K_{i-1}$, it can be shown that this curve consists of N-distinctive and consecutive maxima, that their positions determine the stoichiometry of binding sets (see Appendix).

This conclusion was obtained by considering of these assumptions.

1) $n_{Hi}$ is constant and 2) the binding sets are independent. These assumptions are usually reasonable for surfactant-protein system. However, the deconvolution of the peaks is still a difficult problem.

For overcoming to it, equation (5) is rearranged as follows:

$$RT\theta_i/\upsilon_i = n_{Hi} - n_{Hi}\upsilon_i/g_i \quad (6)$$

For a system with one set of binding sites, it can be suggested that the curve of $[RT\theta/\nu]$ versus $\nu$ should be linear, where the slope, Y and X-intercepts are $-n_H/g$, $n_H$ and

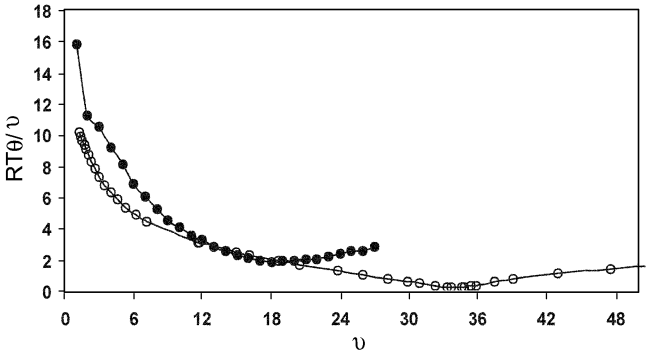


**Figure 3**. The plots of [RT$\theta$/$\upsilon$] versus $\nu$ for binding of CTAB with HSA at pH=7.0, 5 mM phosphate buffer and 27 °C. (●) $10^{-3}$ M and (○) $10^{-4}$ M of NaBr.

*g*, respectively. However, the shape of this curve for multiclasses of binding sites must be more complicated. The plots of [RT$\theta$/$\upsilon$] versus $\upsilon$ for binding of CTAB to HSA are shown in Figure 3.

This curve can be divided to three and two linear parts which represents the existence of three and two binding sets

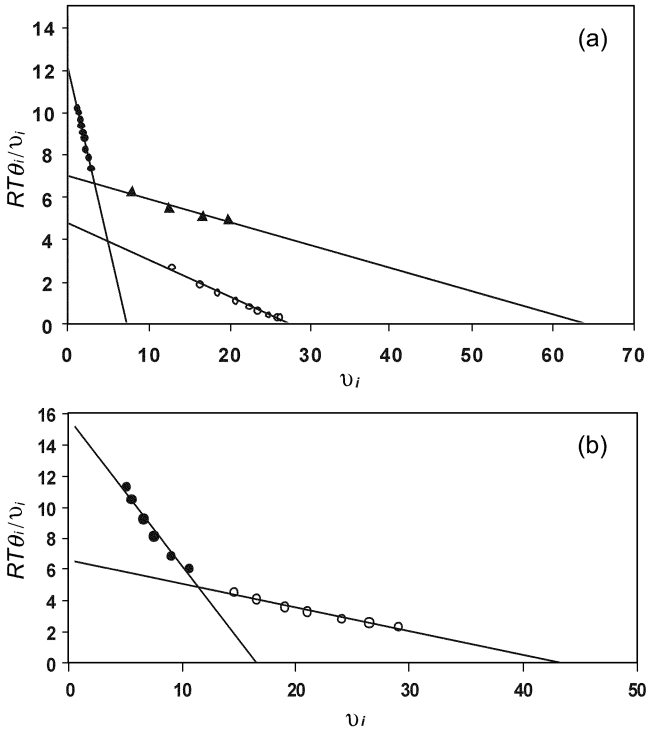


**Figure 4**. The variation of RT$\theta_i$/$\nu_i$ versus $\nu_i$ for interaction of CTAB with HSA at pH 7.0, 5 mM phosphate buffer and 27 °C. (a) [NaBr] = $10^{-4}$ M, (●) first binding set, (○) second binding set, (▲) third binding set (b) [NaBr] = $10^{-3}$ M, (●) first binding set, (○) second binding set.

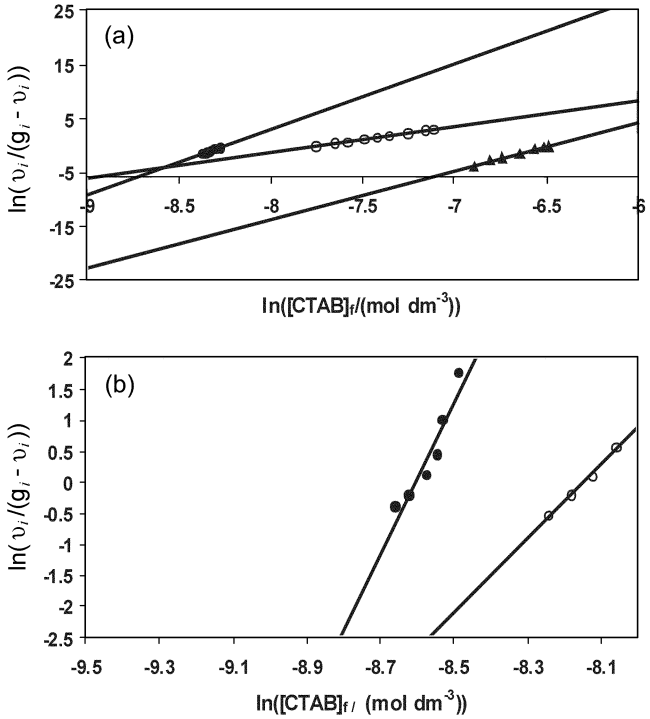


**Figure 5**. The Hill plots for interaction of CTAB with HSA at pH=7.0, 5 mM phosphate buffer and 27 °C. (a) [NaBr] = $10^{-4}$ M, (●) first binding set, (○) second binding set, (▲) third binding set. (b) [NaBr] = $10^{-3}$ M, (●) first binding set, (○) second binding set.

at $10^{-4}$ and $10^{-3}$ M of NaBr, respectively.

The number of binding sites of each set can be estimated from simple extrapolation of each linear part. Figure 4a and b show the precise linear plots of RT$\theta_i$/$\nu_i$ versus $\nu_i$ for each set subsequently.

The slope of this curve is related to $n_{Hi}$ with respect to equation (6). Knowing $n_{Hi}$ and $g_i$, the Hill plots were constructed for estimation of Hill binding constant, $K_i$. Figure 5a and b show the corresponding Hill plots of each binding set, for interaction of CTAB with HSA at $10^{-4}$ and $10^{-3}$ M of NaBr, respectively. The high values of linear correlation coeffiecients of these lines confirm our binding data analysis. The collective values of Hill parameters were listed in Table 1.

The intrinsic Gibbs free energy of binding per mole of CTAB for ith binding set, $\Delta G^{(i)}_{b,\nu}$, can be calculated by the following equation[16]:

$$\Delta G^{(i)}_{b,\nu} = -RTn_{Hi}\ln K_i + RT\,(1-n_{Hi})\ln [CTAB]_f \qquad (7)$$

Figure 6a and b show the variation of $\Delta G^{(i)}_{b,\nu}$ versus ln[CTAB]$_f$ for interaction of CTAB with HSA at $10^{-3}$ and $10^{-4}$ M of NaBr, respectively.

**Table 1**. The collective values of Hill parameters for interaction of CTAB with HSA at pH = 7.0, 5 mM phosphate buffer and 27 °C

| [NaBr] M | $n_{H1}$ | $g_1$ | $K_1$ ($M^{-1}$) | $n_{H2}$ | $g_2$ | $K_2$ ($M^{-1}$) | $n_{H3}$ | $g_3$ | $K_3$ ($M^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| $10^{-4}$ | 12.20 | 7.40 | $3.80 \times 10^3$ | 4.83 | 27.62 | $2.26 \times 10^3$ | 8.34 | 39.8 | $6.53 \times 10^2$ |
| $10^{-3}$ | 12.00 | 12.3 | $5.47 \times 10^3$ | 5.96 | 31.70 | $3.48 \times 10^3$ | – | – | – |

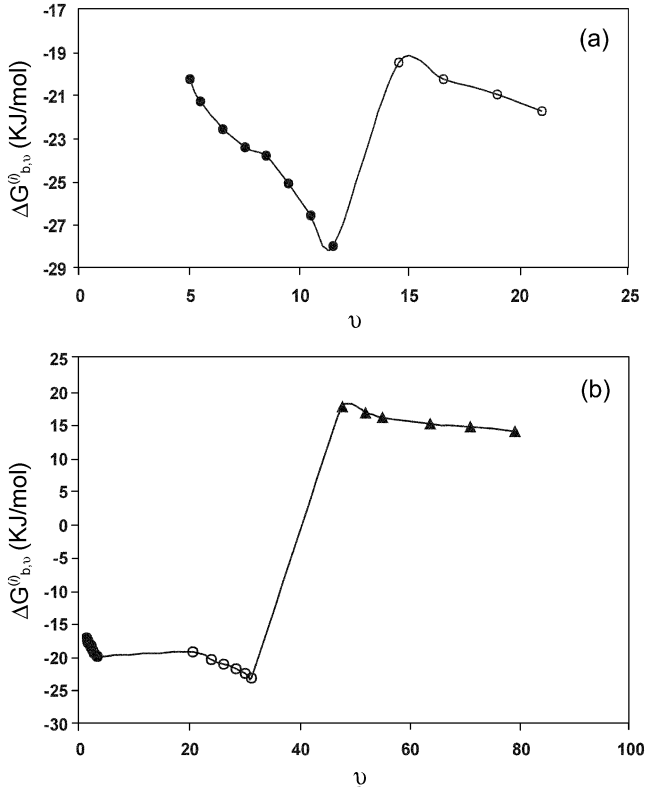


**Figure 6**. The variation of $\Delta G^{(i)}_{b,\upsilon}$ *versus* $\upsilon$ for interaction of CTAB with HSA at pH = 7.0, 5 mM phosphate buffer and 27 °C. (a) [NaBr] = $10^{-3}$ M, ( ● ) first binding set, ( ○ ) second binding set. (b) [NaBr] = $10^{-4}$ M, ( ● ) first binding set, ( ○ ) second binding set, ( ▲ ) third binding set.

## Conclusion

When the Scatchard analysis is applied to protein-surfactant interaction, examples of various kinds of cooperatively and unusual features were found.[22,23] Although, the unusual feature of the Scatchard plot can be correlated to the existence of more than one binding set, the widespread misinterpretation and incorrect usage of non-linear Scatchard plots for surfactant-protein binding experiments are possible.[24] This shortcoming arises from the similarity among the Scatchard plots for systems with various numbers of binding sets. Our proposed model on basis of binding capacity concept removed this shortcoming and determines the number of binding sets. Moreover, this method provides sufficient information for characterization of each binding set. The results for HSA-CTAB interaction represent the existence of two and three binding sets at $10^{-3}$ and $10^{-4}$ M of NaBr, respectively. One mechanism of interaction for ionic surfactant-protein, could be the following: initial strong binding of surfactant ions, at low concentration, occurs to the ionic sites with opposite charge on the protein surface, this may, however, induce protein unfolding thus exposing many more hydrophobic binding sites previously buried in the core of tertiary structure.[13,25] Therefore, there are at least two binding sets in such systems. The first binding set was considered as electrostatic and the second hydrophobic. However, the role of initial hydrophobic interaction in the first binding set has been generally accepted. This interpretation can be successfully applied for HSA-CTAB interaction at $10^{-3}$ M of NaBr. The positive cooperativity was observed in both binding sets which represents the special role of hydrophobic interactions. The three binding set behavior at $10^{-3}$ M of NaBr, can be related to the difference in binding affinity of various ionic binding sites on HSA at this condition. It is well known that HSA consist of three distinct domains in its folded structure that each carrying net charges of -9e (domain I, N-terminal), -8e (domain II) and +2e (domain III, C-terminal).[2] Therefore, all of its negative charges have been located in domain I and II. It is reasonable that each domain is taken as a distinct electrostatic binding set for interaction of CTAB with HSA.

Hence, the first two binding set at $10^{-4}$ M of NaBr can be related to negative ionic charges in domain I and II, and the last to unfolded state of HSA and exposure of hydrophobic binding sites. This interpretation is confirmed by the little difference in binding affinity of first and second binding sets and relative high difference with third binding set (see Figure 6). The reduction of ionic interactions at $10^{-3}$ M of NaBr, caused the difference between binding affinity of these first two sets is reduced, so that both of them behaves as single binding set.

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**Appendix.** If $K_{i-1} >> K_i$, it means that the occupation of ith binding set is not been started until the saturation of (i–1)th binding set. In the other word it can be written :

$$\begin{aligned}
&\upsilon = \upsilon_1,\ \theta = \theta_1 && \text{if} \quad \upsilon \le g_1 \\
&\upsilon = g_1 + \upsilon_2,\ \theta = \theta_2 && \text{if} \quad g_1 < \upsilon \le (g_1 + g_2) \\
&\quad\vdots && \quad\vdots \\
&\upsilon = g_1 + g_2 + \ldots + \upsilon_i,\ \theta = \theta_i && \text{if} \\
&(g_1 + g_2 + \ldots g_{i-1}) < \upsilon \le (g_1 + g_2 + \ldots g_i)
\end{aligned} \tag{8}$$

With respect to equation (5), the derivative of $\theta$ versus $\upsilon$ is as follows :

$$\begin{aligned}
&\left(\frac{\partial\theta}{\partial\upsilon}\right) = \frac{n_{H1}(g_1 + 2\upsilon_1)}{RTg_1} && \text{if} \quad 0 < \upsilon < g_1 \\
&\left(\frac{\partial\theta}{\partial\upsilon}\right) = \frac{n_{H2}(g_2 + 2\upsilon_2)}{RTg_2} && \text{if} \quad g_1 < \upsilon \le (g_1 + g_2) \\
&\quad\vdots && \quad\vdots \\
&\left(\frac{\partial\theta}{\partial\upsilon}\right) = \frac{n_{Hi}(g_i + 2\upsilon_i)}{RTg_i} && \text{if} \\
&(g_1 + g_2 + \ldots g_{i-1}) < \upsilon \le (g_1 + g_2 + \ldots g_i)
\end{aligned} \tag{9}$$

Appling the maximum criteria , it can be shown that :

$$\begin{aligned} \upsilon_{max,1} &= g_1/2 \\ \upsilon_{max,2} &= g_1 + g_2/2 \\ &\cdot \\ &\cdot \\ &\cdot \\ &\cdot \\ \upsilon_{max,i} &= g_1 + g_2 + \ldots g_i/2 \end{aligned} \quad (10)$$

Hence, the binding curve for such system consists of N- consecutive maxima that their positions determine the number of binding sites at each set.

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