Three solutions of the protein solubility problem

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Abstract

Three simple equations are presented, which describe the variation of protein solubility (S) with changes in salt concentration, in terms of either the salt molality (M), the salt activity (a_x), or the water activity (a_w). Each equation yields, essentially independent, estimates of the numbers of salt ions ($\Delta\nu_x$) and water molecules ($\Delta\nu_w$) involved in the dissolution of a mol of the protein. The equations can be used to elucidate the physical significance of the parameters in other empirical equations for protein solubility.

Keywords: equations for protein solubility; protein “salting out” and water binding; salt activity—effect on protein solubility; salt molality—effect on protein solubility; water activity—effect on protein solubility

Protein solubilities have long been known to be very sensitive to salt and hydrogen ion concentrations (Hofmeister, 1888; Cohn, 1925; Green, 1932b; Fox & Forster, 1957; Dixon & Webb, 1961). These effects should throw light on what is happening when protein crystals dissolve and, hence, on possible structural differences between the soluble and crystalline molecules (Rupley, 1968). The solubility curve not only has great theoretical interest, but also has great practical utility (Dixon & Webb, 1961). Unfortunately, the equations that have been proposed to describe the salt effects (Arakawa & Timasheff, 1985) suffer from two serious deficiencies; they hold over only limited ranges for only certain salts, or they contain empirical constants that have no satisfactory physical basis.

The solubilities of small molecules at low salt concentrations can be described by the Debye-Hückel equation (Hückel, 1925), but, when protein solubilities were fit to this same equation (Cohn, 1925; Green, 1932c), unreasonable values were obtained for the adjustable empirical parameters. Cohn (1925) pointed out, however, that for certain salts over a narrow range of high concentrations, protein solubilities could be described by a related empirical equation: $\ln(\text{Solubility}) = \beta - K'_s \mu$, where μ was the ionic strength (for 1,000 g H₂O) and β and K'_s were adjustable parameters. This equation proved to have great utility for systematic protein solubility studies (Dixon & Webb, 1961), but throws little light on what is actually happening. The consensus has been that K'_s is related either to protein hydration (Cohn & Edsall, 1943; Fox & Forster, 1957; Rupley, 1968; Arakawa & Timasheff, 1984) or to the solution surface tension (Melander & Horvath, 1977).

Protein solubility is a thermodynamic quantity and, hence, the salt effects on protein solubility are governed by Wyman's thermodynamic linkage equations (Wyman, 1964). For example, Wyman

showed that when an equilibrium constant (K) is perturbed by a solute X then

$$d \cdot \ln K / d \cdot \ln(a_x) = \Delta\nu_x \quad (1)$$

where a_x is the activity of the solute and $\Delta\nu_x$ is the net change in the number of bound X molecules. This equation enables one to carry out thermodynamic bookkeeping analyses of the stoichiometries of equilibria (Jenkins, 1988, 1989a, 1989b). Tanford subsequently pointed out (Tanford, 1969) that when studying protein interactions at high salt concentrations, it would be more informative to take into account the effects of these high salt concentrations on the binding of water molecules, and he showed that Wyman's equation could be modified, in order to do this, by putting

$$d \cdot \ln K / d \cdot \ln(a_x) = \Delta\nu_x - \Delta\nu_w \cdot N_x / N_w \quad (2)$$

where $\Delta\nu_w$ is the net change in water molecules bound and N_x/N_w the ratio of solute to solvent molecules.

In this article I show that a simple empirical equation, which describes the solubility of a protein over wide ranges of different salt concentrations, with only two adjustable parameters, yields the Wyman-Tanford linkage equation. The two empirical parameters can, therefore, be related to the changes in protein solvation and ion binding when the crystal dissolves.

The reciprocal form of the Wyman-Tanford equation is

$$d \cdot \ln K / d \cdot \ln(a_w) = \Delta\nu_w - (\Delta\nu_x) \cdot N_w / N_x \quad (3)$$

This equation provides an alternative and better method to evaluate ($\Delta\nu_x$) and ($\Delta\nu_w$) from the solubility changes with changes in the water activity (a_w).

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Fig. 1. Variation of salt activities with molalities. The data were derived from Robinson and Stokes (1968). The slopes are given in Table 1.

Theory

Remarkably, for most salts (not MgSO_4), there appears to be, for the concentrations of interest, a simple empirical relationship between salt activity and molality:

$$d(\ln \text{Activity})/d(\ln \text{Molality}) \approx G. \quad (4)$$

In other words the gradient (G) of a plot of $\ln(\text{Activity})$ versus $\ln(\text{Molality})$ is nearly constant (Fig. 1). The constants (Table 1) are approximately 2 for both dissociated salts such as KCl and 2:1 undissociated salts such as $(\text{NH}_4)_2\text{SO}_4$, whereas they are approximately unity for undissociated 2:2 salts such as MgSO_4 .

It is also possible (Fig. 2) to evaluate the molality (M) in terms of a third order polynomial in $\ln(a_x)$.

$$M = P_1 + P_2 \cdot (\ln a_x) + P_3 \cdot (\ln a_x)^2 + P_4 \cdot (\ln a_x)^3. \quad (5)$$

To convert the molalities (M) into water activities there is another empirical expression:

$$\ln(a_w) = -P/(1/(0.018 \cdot M) - Q). \quad (6)$$

Figure 3 illustrates the validity of this empirical equation. Values of the adjustable parameters, P and Q , are summarized in Table 4.

The variation of protein solubility with salt molality

The empirical equation that was found to fit the hemoglobin solubility data of Green in terms of molality (M) is

$$\ln(S) = [(\Delta \nu_x) \cdot \ln(M) - 0.018 \cdot (\Delta \nu_w) \cdot (M)] \cdot G + C \quad (7)$$

where (S) is the solubility/1,000 g water, M is the molality of the salt, $G = d(\ln a_x)/d \cdot (\ln M)$, $\Delta \nu_x$ and $\Delta \nu_w$ are the numbers of salt ions and water molecules involved, and C is an integration constant.

Table 1. Conversion parameters

Salts	$d \cdot \ln a_x / d \cdot \ln M$	P_1^a	P_2^a	P_3^a	P_4^a
NaCl	1.93 ± 0.02	1.56	0.74	0.124	0.006
KCl	1.81 ± 0.01	1.74	0.94	0.214	0.018
$(\text{NH}_4)_2\text{SO}_4$	1.88 ± 0.02	5.71	2.22	0.306	0.015
Na_2SO_4	1.93 ± 0.03	3.32	0.965	0.092	0.0029
K_2SO_4^b	2.01 ± 0.03	2.98	0.867	0.088	0.0030
MgSO_4	1.01 ± 0.00	1.37	-2.23	-0.63	-0.042

^aMolality = $P_1 + P_2 \cdot \ln a_x + P_3 \cdot (\ln a_x)^2 + P_4 \cdot (\ln a_x)^3$.

^bData not shown.

Assuming that $\Delta \nu_x$, $\Delta \nu_w$, and G are all constant, differentiation of this equation, with respect to $\ln M$, gives

$$d(\ln S)/d(\ln a_x) = \Delta \nu_x - 0.018 \cdot \Delta \nu_w \cdot (M). \quad (8)$$

Since $0.018 \cdot (M) = N_x/N_w$, this is identical to the Wyman-Tanford equation (Equation 2).

Equation 7 apparently makes the erroneous prediction that all proteins would be insoluble in pure water. In fact, Equation 7 is only valid over a concentration range where Equation 4 is valid and one can assume that the numbers of bound salt ions and water molecules are invariant. To account for the possible salt dissociation (K) at low concentrations, Equation 7 can be modified to

$$\ln(\text{Solubility}) = [\Delta \nu_x \cdot M \cdot \ln(M)/(K + M) - 0.018 \cdot \Delta \nu_w \cdot (M)] \cdot G + C. \quad (9)$$

Fitting the experimental data to this equation gave values for the putative dissociation constant K of about 10 mM, indicating that the experimental salt concentrations (>0.1 M) were sufficient to saturate the protein.

Fig. 2. The empirical relationship between salt molality and activity. The data were fitted to a third-order polynomial (Equation 5) and the coefficients are given in Table 1.

Fig. 3. An empirical relationship between salt concentrations (M) and water activities (a_w). The lines were drawn for the empirical equation, (Equation 6), $\ln(a_w) = -P/((1/0.018M) - Q)$. Values of the parameters P and Q are given in Table 4.

The variation of protein solubility with the salt activity

Combining the Wyman-Tanford equation (Equation 2) with the empirical polynomial (Equation 5) gives

$$d \cdot \ln(S) / d \cdot (\ln a_x) = \Delta \nu_x - 0.018 \cdot \Delta \nu_w \cdot (P_1 + P_2 \cdot (\ln a_x) + P_3 \cdot (\ln a_x)^2 + P_4 \cdot (\ln a_x)^3). \quad (10)$$

Equation 10 can be integrated to yield

$$\ln(S) = (\Delta \nu_x - 0.018 \cdot P_1 \cdot \Delta \nu_w) \cdot \ln a_x - 0.018 \cdot \Delta \nu_w (P_2 \cdot (\ln a_x)^2 / 2 + P_3 \cdot (\ln a_x)^3 / 3 + P_4 \cdot (\ln a_x)^4 / 4) + C. \quad (11)$$

Because the polynomial parameters (P_n) are known, nonlinear regression analyses give the values for $\Delta \nu_x$, $\Delta \nu_w$, and the integration constant (C).

The variation of protein solubility with the water activity

It is more desirable to analyze the variation of protein solubility with the water activity changes than with the molality changes. This is because salt activities, at the high concentrations of interest, are usually derived from experimentally determined water activities, which involves both numerical integration and also making some questionable ad hoc assumptions (e.g., that the salts are fully dissociated). Furthermore, the resulting equation for protein solubility yields a more reliable value for the change in solvation ($\Delta \nu_w$), which is of primary interest, because it is a dominant factor in protein "salting out."

If $\ln(\text{Solubility})$ is plotted with respect to $\ln(a_w)$, the slopes are given by the reciprocal form of the Wyman-Tanford equation:

$$d \cdot \ln(S) / d \cdot \ln(a_w) = (\Delta \nu_w) - (\Delta \nu_x)(N_w/N_x). \quad (3)$$

Fortunately, there is another good empirical relationship, which can be used (see Fig. 3) to integrate this equation.

$$\ln(a_w) = -P/(N_w/N_x - Q). \quad (6)$$

Combining the two equations, to eliminate N_w/N_x , yields

$$d(\ln(S)) / d(\ln a_w) = (\Delta \nu_w) - (\Delta \nu_x)(-P/\ln(a_w) + Q). \quad (12)$$

Integrating this equation yields

$$\ln(S) = [(\Delta \nu_w) - Q \cdot (\Delta \nu_x)] \ln(a_w) + P \cdot (\Delta \nu_x) \cdot \ln(-\ln(a_w)) + C'. \quad (13)$$

Since the parameters P and Q are known, non-linear regression yields values for $\Delta \nu_x$, $\Delta \nu_w$, and the integration constant C' . For the sulfates, $(\Delta \nu_w) \gg Q \cdot (\Delta \nu_x)$ and, thus, the Q correction term for nonideal solutions (Equation 6) has only a minimal effect.

To estimate the $\Delta \nu_x$ and $\Delta \nu_w$ parameters, Equations 7 and 13 can be readily rearranged into linear forms. For example, Equation 7 yields

$$(\ln(S/S_1)) / (G \cdot \ln(M)) = (\Delta \nu_x + 0.018 \cdot \Delta \nu_w \cdot (1 - M) / \ln(M)) \quad (14)$$

where S_1 is the solubility in 1 molal salt. Similarly Equation 13 yields

$$\begin{aligned} & (\ln(S/S_{0.03})) / \ln(-\ln(a_w)/0.03) \\ &= P \cdot \Delta \nu_x + (\Delta \nu_w - Q \cdot \Delta \nu_x)(\ln(a_w) + 0.03) / \ln(-\ln(a_w)/0.03) \end{aligned} \quad (15)$$

where $S_{0.03}$ is the solubility when $\ln(a_w) = -0.03$. For statistical analyses the nonlinear plots are preferable. The scatter in the linear plots is much greater, due to the fact that they are based on the ratios of two solubility measurements.

Results

Figure 4 shows some of Green's data plotted in the form of $\ln(\text{solubility})$ versus $\ln(\text{salt molality})$. The lines were fitted to Equation 7 by non-linear regression analyses with simple weighting. For the best fit the integration constant (C) was also assumed to be an adjustable parameter. [There are, in fact, only two independently adjustable variables because C is related to the adjustable parameter $\Delta\nu_w$ and the logarithm of the solubility in a 1 M solution ($\ln[S_1] = -0.018 \cdot \Delta\nu_w + C$).] Table 2 summarizes the values found for the numbers of salt ions ($\Delta\nu_x$), water molecules ($\Delta\nu_w$) bound, and the integration constants C . Figure 5 shows the data for ammonium sulfate plotted in the linear form of Equation 14.

Figure 6 shows the solubility data plotted with respect to $\ln(\text{salt activity})$ and fitted by Equation 14. The values of $\Delta\nu_x$, $\Delta\nu_w$, and the integration constant C are given in Table 3.

Figure 7 shows the protein solubility data plotted with respect to the water activities. The data were fitted to Equation 13 and the resulting values for ($\Delta\nu_w$), ($\Delta\nu_x$), and integration constant C' are shown in Table 4. Figure 8 shows the data for ammonium sulfate plotted in the linear form of Equation 15.

Discussion

Figure 1 implies that sulfates are incompletely dissociated. Although it is commonly assumed that they are fully dissociated (e.g., in calculating the ionic strengths and activities of salt solutions), it was shown by early conductometric (Davies, 1927; Onsager, 1927; Righellato & Davies, 1930) and isopiestic analyses (Wishaw & Stokes, 1954) that this was not the case. Resonance Raman spectroscopic studies confirmed the fact that concentrated solutions of sulfuric acid and sulfate salts contained only trace amounts of the sulfate dianion (Young, 1951).

What are the empirical parameters P and Q in Equation 6 and Figure 3? For an ideal solution (Raoult's Law) $a_w = N_w/(N_x + N_w) = N_w/(\nu N_x + N_w)$ for a salt forming ν ions per mol in solution. Bjerrum (1920) and Stokes and Robinson (1948) pointed out that one should also include a correction for the number of mol

Table 2. Hemoglobin solubility changes with salt molality

Salts	$\Delta\nu_x$	$\Delta\nu_w$	Constant
NaCl	0.22 ± 0.02	0.60 ± 1.1	5.30 ± 0.07
KCl	0.36 ± 0.01	12.2 ± 0.50	5.63 ± 0.03
$(\text{NH}_4)_2\text{SO}_4$	0.49 ± 0.05	125 ± 4	6.47 ± 0.16
Na_2SO_4	0.27 ± 0.03	146 ± 6	5.87 ± 0.21
K_2SO_4^a	0.23 ± 0.05	121 ± 15	5.49 ± 0.31
MgSO_4	1.10 ± 0.10^b	173 ± 7^b	7.04 ± 0.19^b

^aData not shown.

^bNumbers suspect because Equation 4 was not valid.

of water (H) sequestered by each mol of the anhydrous salt by writing $a_w = (N_w - H \cdot N_x)/(\nu N_x + N_w - H \cdot N_x)$. It follows that

$$-\ln(a_w) = \ln(1 + \nu \cdot N_x/(N_w - H \cdot N_x)) \quad (16)$$

$$\approx \nu/(N_w/N_x - H) \quad (17)$$

because this is a small number. Comparison with the empirical equation (Equation 6) suggests that P should be the number of ions produced per mol of salt (ν), and Q might be the number of mol of water sequestered per mol of anhydrous salt (H). Because of ion interactions, P is less than the possible number of ions. For example, $P \approx 2$ for both monovalent dissociating salts such as chlorides and also for sulfates because, at high concentrations, they do not dissociate completely into sulfate dianions. Unfortunately, the values of Q , required for fitting, give chemically unreasonable values for the numbers of water molecules (H) that might react. For example, fitting some sulfates yields large *negative* numbers for the hydration of the salt ions! Because more work clearly needs to be done, it is advisable to consider Equation 6 merely a useful empirical summary of the variation of the water activity with molality.

Fig. 4. Effect of salt concentrations on the solubility of crystalline carboxyhemoglobin. Data replotted from Green (1932a, 1932b). The lines are fitted by Equation 7. The fitting parameters are given in Table 2.

Fig. 5. The ammonium sulfate data of Figure 4 plotted in a linear form according to Equation 14. The ordinate intercept is equivalent to $\Delta\nu_x = 0.47 \pm 0.10$ and the slope is equivalent to $\Delta\nu_w = 126 \pm 6$.

Fig. 6. The solubility of carboxyhemoglobin in different salt solutions plotted with respect to the salt activity. These are the same data of Green as in Figure 4. The lines are fitted to Equation 10 and the fitting parameters are given in Table 3.

The $\Delta\nu_x$ parameters, in Tables 2–4, indicate that less than one salt ion is bound. By an extension of Wyman's equation (Jenkins, 1989a, 1989b) one can show that the "ions bound" ($\Delta\nu_x$), in fact, the sum of the number of cations and anions. That so few are bound probably results from the fact that Green chose to make her study near to the isoelectric pH for hemoglobin, where one might anticipate that few ions would bind.

The sulfates give a consistent value of about 130 for the numbers of water molecules bound, but this is two orders of magnitude greater than that with chlorides. This discrepancy arises from the well known effect (Cox & Schumaker, 1961; Bull & Breese, 1970; Kuntz & Kauzmann, 1974; Arakawa & Timasheff, 1982) that sulfates are not bound but induce the binding of water molecules, whereas chlorides do not cause such a discrimination. Tanford (1969) pointed out that the slope $d(\ln K)/d(\ln a_x)$ is, in reality, the preferential binding of the salt solute relative to the solute/solvent ratio found in the solution distant from the macromolecule. Thus, the positive slopes in Figure 4 indicate the preferential binding of salt ions and the negative slopes, observed at high concentrations of "salting out" salts, indicate the preferential binding of water molecules.

As a first approximation the ratio $d \cdot \ln(a_s)/d \cdot \ln(M)$ was assumed to be constant. However, if one accepts the validity of the

Table 3. Hemoglobin solubility changes with salt activity

Salts	$\Delta\nu_x$	$\Delta\nu_w$	Constant
NaCl	0.23 ± 0.02	0.89 ± 2.04	5.47 ± 0.05
KCl	0.36 ± 0.01	12 ± 0.05	5.31 ± 0.01
$(\text{NH}_4)_2\text{SO}_4$	0.56 ± 0.05	132 ± 4	-20.4 ± 0.5
Na_2SO_4	0.26 ± 0.03	148 ± 6	-16.4 ± 0.7
K_2SO_4^a	0.23 ± 0.04	121 ± 13	-11.5 ± 1.5
MgSO_4	0.35 ± 0.12	90 ± 6	-7.87 ± 24

^aData not shown.

Table 4. Hemoglobin solubility changes with water activity

Salts	P^a	Q^a	$\Delta\nu_x$	$\Delta\nu_w$	Constant
NaCl	1.78	2.81	0.22 ± 0.02	0.2 ± 1.2	6.66 ± 0.16
KCl	1.74	1.40	0.36 ± 0.01	11.3 ± 0.4	7.71 ± 0.06
$(\text{NH}_4)_2\text{SO}_4$	1.98	-1.53	0.51 ± 0.051	128 ± 4	10.0 ± 0.5
Na_2SO_4^b	2.20	-6.66	0.27 ± 0.04	148 ± 8	8.08 ± 0.52
K_2SO_4^b	2.32	-13.3	0.22 ± 0.05	120 ± 15	7.34 ± 0.74
MgSO_4	0.87	9.79	0.62 ± 0.08	97 ± 4	7.84 ± 0.38

^aFor Equation 6, $\ln(a_w) = -P/(1/(0.018 \cdot M) - Q)$.

^bData not shown.

empirical equation $\ln(a_w) = -P/(N_w/N_x - Q)$, then from the Gibbs-Duhem relationship, because $d \cdot \ln(M)/d \cdot (M) = 1/(M) = 1/N_x$

$$\begin{aligned} d \cdot \ln(a_x)/d \cdot \ln(M) &= (d \cdot \ln(a_w)/d \cdot \ln(M))(N_w/N_x) \\ &= -N_w \cdot d \cdot \ln(a_w)/d \cdot (M). \end{aligned}$$

It follows that $d \cdot \ln(a_x)/d \cdot \ln(M) = P/(1 - Q \cdot N_x/N_w)^2$. The limiting value for the ratio at low salt concentration ($N_x = 0$) is thus equal to P . Although N_x/N_w is a small number, one can conclude that if Q is negative, then $d \cdot \ln(a_s)/d \cdot \ln(M)$ will decrease slightly with concentration, whereas if Q is positive, this slope will increase slightly with concentration (see Tables 1 and 4).

By equating my Equation 7 to other empirical equations it is possible to evaluate their empirical coefficients. For example, differentiating Equation 7, with respect to molality:

$$d \cdot \ln(\text{Sol.})/d \cdot (M) = (\Delta\nu_x/M - 0.018 \cdot \Delta\nu_w)G$$

shows how the slope term (K'_s) in Cohn's equation is related to both salt and water binding parameters. Only at high salt concen-

Fig. 7. The effects of salts on the solubility of carboxyhemoglobin, plotted with respect to the water activity. The lines are drawn according to Equation 13, and the fitting parameters are given in Table 4.

Fig. 8. The solubility in ammonium sulfate solutions plotted with respect to the water activities in a linear form, according to Equation 15.

trations (M) $\gg \Delta\nu_x/(0.018 \cdot \Delta\nu_w)$ does K'_s depend primarily on the water solvation term $\Delta\nu_w$.

Green (1932c) fitted her data to the simple empirical equation,

$$\log(\text{solubility}) = 1.3 + 1.6\sqrt{C} - k_o \cdot (C)$$

where ($C = F \cdot M$) was the "equivalent concentration" (i.e., M for univalent salts, such as NaCl; $2M$ for both univalent/bivalent salts such as $(\text{NH}_4)_2\text{SO}_4$ and bivalent/bivalent salts such as MgSO_4 ; and $3M$ for trivalent salts such as sodium citrate). Although it received little recognition, this equation is of interest because it fit all the data with only one salt dependent parameter k_o ; Cohn's equation, in contrast, fitted only a few of the data points with two salt dependent parameters! Because analytically (i.e., by curve fitting) $\sqrt{C} = 0.76 + 0.24(C) + 0.23 \ln(C)$, Green's equation can be rewritten:

$$\log(S) = 1.3 + 1.6(0.76 + 0.24(C) + 0.23 \ln(C)) - k_o \cdot (C).$$

Equating this equation with Equation 7 shows that her empirical salt-dependent parameter (k_o) was linearly related solely to the number of water molecules bound.

$$k_o = 0.38 + 0.018 \cdot G \cdot \Delta\nu_w / (2.3F).$$

What is noteworthy about the equations in this article is their generality. They apply to other crystalline proteins, to amorphous protein precipitates, and to other protein precipitants, such as polyethyleneglycol. Furthermore, they apply to other equilibria such as protein conformational changes, protein associations, and the binding of ligands to macromolecules.

Experimental data and methods

The data used for analyses were published in a classical series of papers by Green (1932a, 1932c) concerning the effects of salts on the solubility of crystalline horse carboxyhemoglobin. Her experimental data were published in both tabular form and in figures.

Fortunately, she cited both molarities and molal ionic strengths (μ) in her tables. The molalities are readily derived from the latter. They were confirmed by using published tables (Wolf et al., 1972). I used the data from two of her tables; Table 5 of Green (1932a) and Table 1 of Green (1932c). These same data have frequently been used in textbooks and for other analyses of protein solubility (Melander & Horvath, 1977). One unusually favorable aspect was that a crystalline protein was used and the sample of crystals was either prepared or equilibrated with the solvent of interest, to avoid having mixtures of different ions present. She also carried out the studies close to the pH of minimum solubility so that possible pH perturbations were minimized (Green, 1932b).

To use the Wyman-Tanford equation it is necessary to know how the salt and water activities vary with the salt molality. There are extensive compilations of such data (Wishaw & Stokes, 1954; Robinson & Stokes, 1968; Goldberg, 1981) based upon isopiestic measurements of the water activity and the Gibbs-Duhem relationship between changes in water and salt activities. The water activities were derived from osmotic coefficients, obtained by interpolation, whereas the salt activities were derived from mean molal ion activity coefficients, obtained by interpolation.

Non-linear regression analyses, and the preparation of the illustrations, were carried out with the GraFit software program (Leatherbarrow, 1992).

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