Solution Properties of Phycocyanin. I. Studies of Dissociation-Association by Sedimentation Measurement*

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The dissociation-association properties of phycocyanin, isolated from the red algae, "*Porphyra tenera*," have been investigated by sedimentation velocity measurements at pH 6.8 and 5.4 and at the ionic strengths of 0.1 and 0.2. The sedimentation coefficient data indicated that the predominant component in the phycocyanin solution was a trimer at pH 6.8 and a hexamer at pH 5.4. The concentration dependence of the sedimentation coefficient for various hypothetic systems of the dissociation-association equilibrium of phycocyanin was simulated by a computer model. It was reasonable to consider, from the simulation, that the dissociation-association system of phycocyanin was trimer$\rightleftarrows$monomer at pH 6.8 and hexamer$\rightleftarrows$monomer at pH 5.4. The dissociation constant was calculated for each system. The value of the dissociation constant was 0.15×10^{-4} (g/dl)^2 at pH 6.8 and an ionic strength of 0.1; 0.13×10^{-4} (g/dl)^2 at pH 6.8 and an ionic strength of 0.2; 0.5×10^{-13} (g/dl)^5 at pH 5.4 and an ionic strength of 0.1 and 0.1×10^{-13} (g/dl)^5 at pH 5.4 and an ionic strength of 0.2. The dissociation constants decrease with an increase in the ionic strength in solution.

Phycocyanin is an accessory photosynthetic chromoprotein obtained from red- and blue-green algae. The properties of phycocyanin have been studied by many investigators. Svedberg and Katsurai[1] and Svedberg and Eriksson[2] measured the molecular weight of the protein under various pH conditions by the sedimentation equilibrium method; they found that the molecular weight of phycocyanin changed reversibly according to the pH change of the solution. Recently, Hattori and Fujita[3] and Berns *et al.*[4] confirmed this phenomenon by ultracentrifugation and spectrometry. It was showen that the reversible aggregation of this protein depended upon the ionic strength and the temperature of the solution.[5-7] It has also been reported by Berns *et al.*[8] that phycocyanin decomposed into many smaller units in the presence of urea or sodium dodecyl sulfate, and that the minimum molecular weight of phycocyanin was 30000. Scott and Berns.[7] claimed that the molecular weight of the phycocyanin aggregates were 90000, 180000, or 360000. From these observations, it has been presumed that the aggregates of phycocyanin are a trimer, a hexamer and a dodecamer, and also that the dissociation-association equilibrium was attained between these higher aggregates and lower aggregates or a monomer in the solution.

Hattori *et al.*[6] obtained the equilibrium constant from their measurements of the concentration dependence of the extinction coefficient of the phycocyanin solution. Scott and Berns[7] observed two or more peaks in the schlieren patterns obtained by the sedimentation velocity measurement of phycocyanin from *Plectonema calothricoides*, and calculated the equilibrium constant using the relative areas under these peaks.

Adams *et al.*[9-12] have published a theory of the association-dissociation of molecules in an ultracentrifugal field and have derived a method for evaluating the equilibrium constant from the sedimentation equilibrium experiment. They applied this theory to the study of the self-association of β-lactoglobulin B in an acid solution[13] and showed that the experimental results could easily be explained by this theory.

In order to investigate the molecular shape and size of phycocyanin, which undergoes the dissociation-association reaction according to the environmental changes in the solution, it is necessary to estimate the dissociation-association constant of this protein. Adams' method is not always a convenient one for obtaining such preliminary knowledge as described above. If it is possible to estimate the equilibrium constant for the dissociation-association of phycocyanin by using the sedimentation transport method, such an experiment would, however, be quite useful.

In this paper we will attempt to estimate the equilibrium constant by using the sedimentation velocity method, and also to presume the state of the equilibrium of phycocyanin in solution. Moreover, we wish to contribute to the knowledge about the solution properties of the phycocyanin.

Experimental

Preparation. The phycocyanin used in this study was obtained from dried *Porphyra tenera* by repeating the precipitation with ammonium sulfate. The details of this preparation were reported by Fujiwara.[14] The outline of this procedure is presented in Fig. 1. The purity of phycocyanin was ascertained by measurements of its electrophoretic behavior, while the absorption spectra of this protein solution were measured at pH 6.8 and at an ionic strength of 0.1. As is shown in Fig. 2, the ratio of extinction at 620 nm and 280 nm was greater than 4.0. This value agreed with that obtained by earlier investigators[3,15,16] for pure phycocyanin. The purified phycocyanin was stored at 5 °C under 40% saturated ammonium sulfate. The sample of phycocyanin was dissolved in water and dialyzed against a phosphate or acetate buffer at 5 °C. Dialysis was continued for at least 48 hr, with an occasional change of the external solution.

Determination of Concentration. The protein concentrations were determined by weighing the substances after they had been dried at 105 °C for 24 hr; they were also determined by the semi-micro-Kjeldahl method. The value of the concentrations obtained by the different methods

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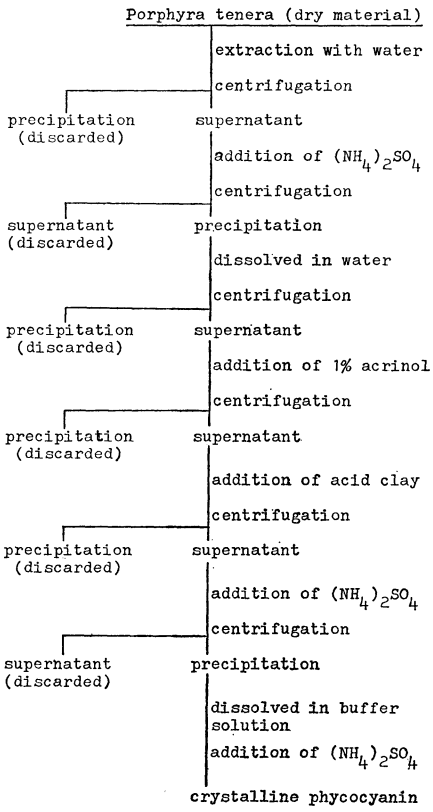


Fig. 1. Flow-sheet of preparation of phycocyanin.

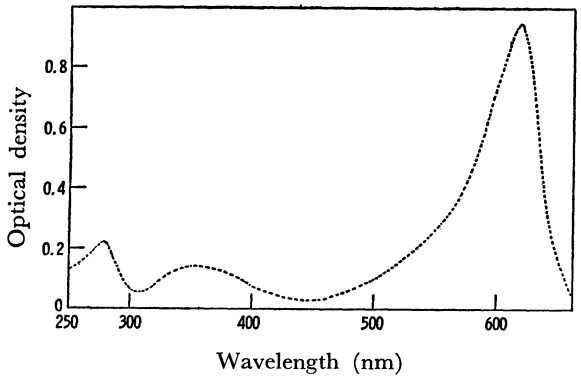


Fig. 2. Absorption spectrum of aqueous solution of phycocyanin.

agreed with each other within the limits of experimental error.

Ultracentrifugation. The sedimentation velocity experiments were performed with a Hitachi Model UCA-1 ultracentrifuge with a schlieren optical system; double sectorial cells were used. In all runs the rotor speed was set at 47,300 rpm, and the temperature was maintained at 25 °C.

The sedimentation coefficient, s, is represented by the following equation:

$$s = \frac{\ln(r_p/r_m)}{\omega^2(t-t_0)},$$

where r_p is the boundary position measured from the second moment of the refractive index gradient; r_m, the position of the meniscus; ω, the angular velocity; t, the time, and t_0, the initial time correction. In general, if the shape of the schlieren pattern is of a Gaussian type, $r_p=r_h$, where r_h is the position of the maximum of the refractive index gradient. In our experiment, the schlieren patterns were satisfactorily considered to be Gaussian, as is shown in Fig. 3; the position of the maximum of the refractive index gradient dose not differ significantly from the boundary position obtained. For example, the calculated values of r_p and r_h were 6.362 cm and 6.363 cm respectively 30 min after reaching a constant speed of 47300 rpm, in the sedimentation velocity measurement of phycocyanin at pH 6.8 and at I (ionic strength) of 0.1. The position of the maximum of the refractive index gradient was, then, used in place of the boundary position for the evaluation of the sedimentation coefficient throughout this study. The position of maximum of the refractive index was measured using a Sinko-VF-12 projector.

Estimation of the Equilibrium Constant for Dissociation-Association Reactions. The sedimentation coefficient for a system containing many species is represented by the following equation:

$$s = \frac{\sum s_i c_i}{\sum c_i}. \quad (1)$$

where c_i and s_i are the weight concentration(g/dl) and the sedimentation constant for the i-th species respectively. The equilibrium constant for the i-mer$\rightleftarrows$monomer($M_i \rightleftarrows iM_1$) system, K_i, is defined by using the mass-action equation:

$$K_i = \frac{{c_1}^i}{c_i}. \quad (2)$$

The total concentration, c, is given by the following equation:

$$c = \sum c_i. \quad (3)$$

Thus, Equation (1) is rewritten in the form:

$$s = \frac{\sum ({c_1}^i/K_i)s_i}{c}. \quad (4)$$

The concentration dependence of the sedimentation coefficient is usually shown as follows:

$$s_i^* = s_i^0(1-\alpha_i), \quad (5)$$

$$s^* = \frac{\sum ({c_1}^i/K_i)s_i^0(1-\alpha_i)}{c}, \quad (6)$$

where s^* is an apparent sedimentation coefficient and s^0, the sedimentation coefficient at an infinite dilution. Moreover α_i is $k_{s_i}c_i$, where k_{s_i} is the concentration-dependence coefficient of the i-th species. Now, by assuming that α_i is constant for all species, the following equation is obtained from Eq. (6):

$$s^* = \left[\frac{\sum ({c_1}^i/K_i)s_i^0}{c}\right](1-k_s c). \quad (7)$$

Thus the equilibrium constant, K_i, can be evaluated if the values of s_i^0, c_1, s^*, and k_s are known.

The sedimentation coefficient of the phycocyanin aggregates, s_i^0, was determined by extrapolating the linear plot over a limited region at higher concentrations to an infinite dilution, while the concentration-dependence coefficient, k_s, was determined from the slope according to Eq. (5). The sedimentation coefficient of the monomer, s_i^0, was assumed to be 3.2 S, a value reported by Hattori *et al.*[6] Then K_i can be computed by assuming the value of c_1. In practice, the value of the right-hand side of Eq. (7) are calculated by assuming both the values of c_1 and K_i, and then compared with the s^* obtained experimentally. The values of c_1 and K_i were systematically changed, and the most probable value of K_i was decided according to where the standard deviation of the difference between the calculated s^* and the observed s^k reaches its minimum. The TOSBAC-3400 computer was used for the calculations.

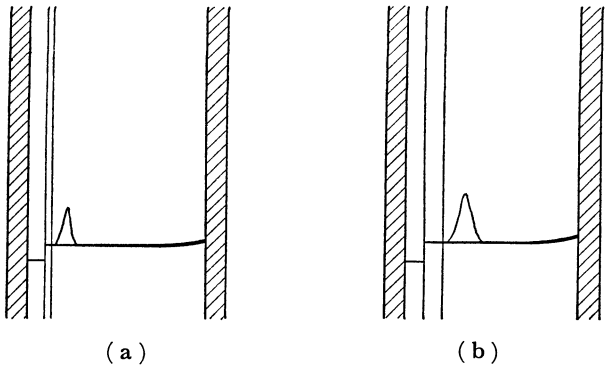
Results and Discussion

Some investigators[6,7,15-17] have observed two or more peaks in the sedimentation patterns of a phycocyanin solution under various values of pH and the ionic sterngth. However, all of our sedimentation patterns showed a single peaks, as is shown in Fig. 3. This discrepancy seems to be caused by the sources of phycocyanin being different from one another.

Scott and Berns[7] estimated the equilibrium constant using the relative areas under the peaks of the schlieren patterns, but in our experiments this procedure is inapplicable to the estimation of the equilibrium constant. Therefore, the following treatment was necessary for the estimation of the equilibrium constant.

Figures 4—7 show the sedimentation coefficients of phycocyanin as a function of the protein concentration at pH 6.8 and 5.4 and at I values of 0.1 and 0.2. In these figures, the calculatted lines for the dissociation-association equilibria are given. The variation of s^* with the concentration shows a tendency to increase with a decrease in the concentration in the higher-concentration region, while it tends to decrease near a zero concentration. These results show that the protein molecules associate in the higher-concentration region. This behavior is similar to that of β-lactoglobulin or γ-G-globulin.[18]

Figure 4 shows the results at pH 6.8 and at $I=0.1$. From the plots in Fig. 4, the s_t^0 of 6.3 S and the k_s of 0.30 were obtained. Since the s_t^0 value agreed with the value reported for the trimer,[6,17] we assumed that this dissociation-association equilibrium was the trimer$\rightleftarrows$monomer ($M_3\rightleftarrows 3M_1$) system. The calculated line A is that for the $M_3\rightleftarrows 3M_1$ system. In order to know whether or not the hexamer species exist in the solution, the (hexamer+trimer)$\rightleftarrows$monomer($M_6+M_3\rightleftarrows 9M_1$) system was calculated as a trial. Line B is that calculated for the system. As is shown in Fig. 4, the experimental results can be well represented by the calculated line A. The hexamer could not be considered to exist in the solution. The equilibrium constant for the $M_3\rightleftarrows 3M_1$ system, K_3, was decided to be 0.15×10^{-4} (g/dl)^2.



(a) (b)

Fig. 3. Sedimentation patterns of phycocyanin.
(a): at pH 6.8, ionic strength 0.1; phosphate buffer, 26 min after reaching constant speed, (b): at pH 5.4, ionic strength 0.1; acetate buffer, 28 min after reaching constant speed.

The results at pH 5.4 and $I=0.1$ are shown in Fig. 5; values of 10.8 S for s_t^0 and 0.30 for k_s were obtained from the results. The s_t^0 value agreed with the value which was reported for the hexamer.[6,7,15,16] This result shows clearly that the state of the dissociation-association equilibrium of the protein is closely related to the pH change in the solution. It was assumed that this dissociation-association equilibrium was the hexamer$\rightleftarrows$monomer($M_6\rightleftarrows 6M_1$), hexamer$\rightleftarrows$trimer($M_6\rightleftarrows 2M_3$), or $M_6+M_3\rightleftarrows 9M_1$ system. The calculated line C is that for the $M_6\rightleftarrows 6M_1$ system; line D, that for the $M_6+M_3\rightleftarrows 9M_1$ system, and line E, that for the $M_6\rightleftarrows 2M_3$ system. It is shown in this figure that the calculated line C for the $M_6\rightleftarrows 6M_1$ system almost coincides with the experimental points, while the other two lines show a larger deviation from the results obtained experimentally. The value of 0.5×10^{-13} (g/dl)^5

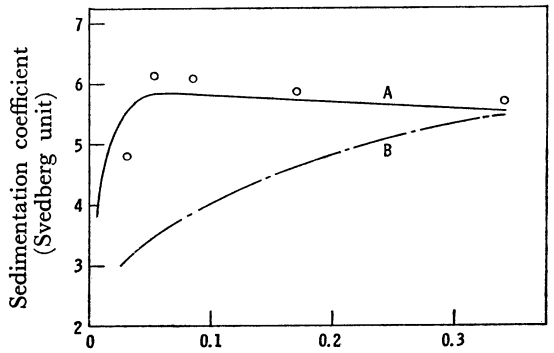


Fig. 4. Plots of sedimentation coefficients *versus* phycocyanin. Concentration in phosphate buffer solution at pH 6.8 and ionic strength 0.1, and calculated lines for the assumed equilibrium systems.
○: experimental points, line A: for the trimer$\rightleftarrows$monomer system, line B: for the (hexamer+trimer)$\rightleftarrows$monomer system.

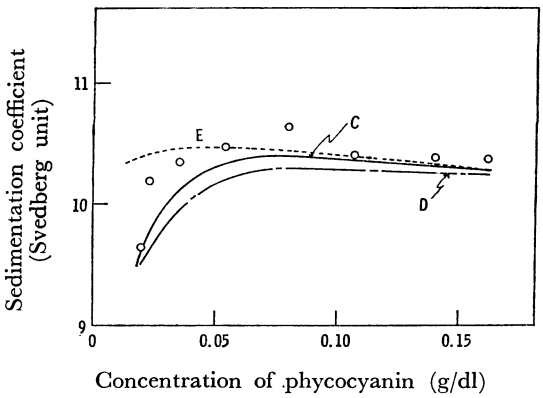


Fig. 5. Plots of sedimentation coefficients *versus* phycocyanin. Concentration in acetate buffer solution at pH 5.4 and ionic strength 0.1, and calculated lines for the assumed equilibrium systems.
○: experimental points, line C: for the hexamer$\rightleftarrows$monomer system, line D: for the (hexamer+trimer)$\rightleftarrows$monomer system, line E: for the hexamer$\rightleftarrows$trimer system.

for the $M_6 \rightleftarrows 6M_1$ dissociation constant, K_6, was obtained.

In order to determine the effect of the ionic strength on the dissociation-association, sedimentation-velocity experiments were also carried out at $I=0.2$ under the same pH condition as before. Some investigations with regard to the effect of the ionic strength on the dissociation-association of phycocyanin have been reported by several authors,[6,7] and it has been found that the quantity of aggregates increased with an increase in the ionic strength of the solution. However, these studies have been done in the lower ionic strength region.

Figure 6 shows the results at pH 6.8 and at $I=0.2$. From the results, the s_i^0 of 6.5S and k_s of 0.16 were obtained. This s_i^0 value agreed with that obtained $I=0.1$ and pH 6.8 in this work. It seemed reasonable to assume that the dissociation-association equilibrium was the $M_3 \rightleftarrows 3M_1$ or the $M_6+M_3 \rightleftarrows 9M_1$ system. This assumption was clearly the same as that at pH 6.8 and at $I=0.1$. The calculated line F is that for the $M_3 \rightleftarrows 3M_1$ system, while line G is that for the $M_6+M_3 \rightleftarrows 9M_1$ system. As is shown in Fig. 6, it was clear that the hexamer did not exist in the solution. The equilibrium constant for the $M_3 \rightleftarrows 3M_1$ system, K_3, was decided to be 0.13×10^{-4} (g/dl)^2. At the same pH, K_3 of 0.4×10^{-4} (g/dl)^2, which acts for the dissociation of phycocyanin in the 0.01 M phosphate buffer solution ($I=0.04$), and K_3 of 1.0×10^{-4} (g/dl)^2 in the 0.001 M phosphate buffer solution(I 0.004) were reported by Hattori *et al.*[6], using an absorption method. As has been described above, K_3 of 0.15×10^{-4} (g/dl)^2 for the phycocyanin solution was obtained in this work at the same pH and at $I=0.1$. These four values for K_3 under the different conditions of ionic strength show a tendency for the equilibrium constant to decrease with an increase in the ionic strength.

Figure 7 shows the results at pH 5.4 and at I=0.2; the s_i^0 of 11.3 S and the k_s of 0.24 were obtained. The s_i^0 value agrees with s_i^0 of 10.8 S which was obtained at $I=0.1$ and at pH 5.4 in this work. As before, this dissociation-association equilibrium was assumed to be $M_6 \rightleftarrows 6M_1$, $M_6+M_3 \rightleftarrows 9M_1$, or $M_6 \rightleftarrows 2M_3$ system. The calculated line H is that for the $M_6 \rightleftarrows 6M_1$ system, line I, that for the $M_6+M_3 \rightleftarrows 9M_1$ system, and line J, that for the $M_6 \rightleftarrows 2M_3$ system. Figure 7 shows that both the H and I calculated lines seem to be in satisfactory agreement with the experimental points. However, the concentration of the trimer, which was calculated in the process of the computation of K_i on the assumption of the $M_6+M_3 \rightleftarrows 9M_1$ system, was negligibly small. Therefore, it seems reasonable to say that the $M_6 \rightleftarrows 6M_1$ equilibrium was attained in the solution at pH 5.4 and at $I=0.2$. The equilibrium constant for the $M_6 \rightleftarrows 6M_1$ system, K_6, was decided to be 0.1×10^{-13} (g/dl)^5. The value is smaller than that obtained in this work at pH 5.4 and at I=0.1. It is found that the increase in the ionic strength relates to the decrease in the equilibrium constant; this result is identical with that obtained at pH 6.8. Hattori *et al.*[6] have explained that an increase in ionic strength results in a reduction of the electrostatic repulsive force of phycocyanin particles, and that this leads to the association of the monomer. The experimental results obtained in this study support their conclusion.

The association-dissociation equilibrium system of phycocyanin was suggested to be either the $6M_1 \rightleftarrows 2M_3 \rightleftarrows M_6$ system or two parallel reactions, that is, the $3M_1 \rightleftarrows M_3$ and the $6M_1 \rightleftarrows M_6$ system.[6,7] Berns *et al.*[19] proposed that the equilibrium system involved three parallel reactions, that is, the $3M_1 \rightleftarrows M_3$, the $6M_1 \rightleftarrows M_6$, and the $2M_3 \rightleftarrows M_6$ systems. In our experiments, the value of the equilibrium constant was small under the pH conditions of 6.8 and 5.4. Thus, it seems reasonable to assume that the dissociation-association equilibrium of phycocyanin under the conditions in this study inclines toward an one-side extreme (aggregate).

The concentration-dependence coefficients, k_s, of these solutions were found to be 0.30 (pH 6.8, $I=0.1$),

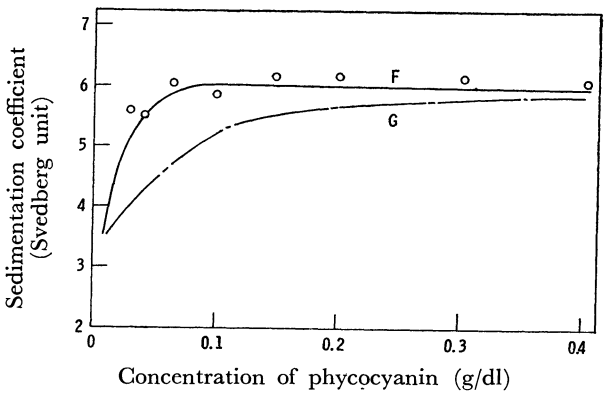


Fig. 6. Plots of sedimentation coefficients *versus* phycocyanin. Concentration in phosphate buffer solution at pH 6.8 and ionic strength 0.2, and calculated lines for the assumed equilibrium systems.
○: Experimental points, line F: for the trimer⇌monomer system, line G: for the (hexamer+trimer)⇌monomer system.

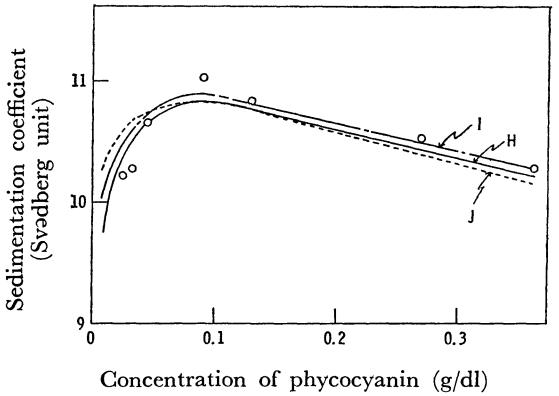


Fig. 7. Plots of sedimentation coefficients *versus* phycocyanin. Concentration in acetate buffer solution at pH 5.4 and ionic strength 0.2, and calculated lines for the assumed equilibrium systems.
○: experimental points, line H: for the hexamer⇌monomer, line I: for the (hexamer+trimer)⇌monomer system, line J: for the hexamer⇌trimer system.

0.30 (pH 5.4, I=0.1), 0.16 (pH 6.8, I=0.2), and 0.24 (pH 5.4, I=0.2). As with the k_s values of many other proteins, these values were small. Weirich *et al.*[20] have published a basic theory of the sedimentation coefficient of self-association species and described a method of estimating the sedimentation coefficient using the equilibrium constant, K_i, and the monomer concentration, c_1, obtained from sedimentation-equilibrium, light-scattering, or osmotic-pressure experiments on the same self-associating solute. Our study was aimed at estimating the equilibrium constant of the dissociation-association equilibrium system from the results of the sedimentation-velocity experiment. Since a sedimentation coefficient is not an equilibrium quantity, the equilibrium constant derived in this study should be considered to be only approximate values. Nonetheless, the dissociation constants obtained in this work are in fair agreement with those constants previously reported.[6] In view of the fact that the sedimentation-velocity measuement requires only a short time, it can be said that the procedure in this paper is a convenient method for the study of the dissociation-association system of a protein.

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