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Effects of Third Component on Hydrophobic and Hydrophilic Moities of Tryptophan in Aqueous Solution. Approach to Understanding of Denaturation of Globular Protein[1,2]

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The solubility properties of tryptophan were observed in various solutions of the denaturants for β-lactoglobulin reported by Tanford, *et al.*, and the effects of such 3rd component on the hydrophobic and the hydrophilic moieties of solute molecule was discussed on the basis of the enthalpy of mixing. As a result, it seemed possible to classify the denaturants into three types according to the balance of the effects on hydrophobic and hydrophilic moieties of tryptophan resulting in a change of solubility.

In comparison with the existing data, the same classification might be extended to the denaturants according to their effects on the denaturation of β-lactoglobulin, and it was established that the solubility of tryptophan or the denaturation of β-lactoglobulin varies with the effects of additive (or denaturant) on hydrophobic and hydrophilic moieties of molecule, where the decrease in enthalpy of mixing is considered to be a predominant factor to result in the increase in solubility of tryptophan or to result in the denaturation of β-lactoglobulin.

Denaturations of protein by solvents or denaturants have been investigated in various ways.[4—8] Each segment of protein molecule consists of hydrophobic and hydrophilic moieties, and it is significant to take the interactions of denaturant with both moieties into consideration of denaturation phenomena.[9] Tanford considered that the hydrophobic side chains in a protein molecule which are shielded from the surrounding liquid come on the surface of the molecule in contact with the liquid when the denaturation takes place.[4] In this regard, the denaturation of protein is related with the thermodynamic functions of the transfer of hydrophobic side chains from the surrounding water to the inside of molecule. It has been accepted from the existing views on hydrophobic bonding[10] that such a transfer as described above may be forced with the increase of entropy through the destruction of iceberg around the hydrophobic side chains, though the destruction of iceberg is considered to be an enthalpy effect according to Shinoda and Fujihira's new view.[11]

In the previous paer,[1] the increase in solubility of tryptophan upon addition of urea was explained on the consideration of the decrease in enthalpy of mixing of the hydrophobic moiety of tryptophan with the solvent, as was derived from Shinoda and Fujihira's new view.[11]

1) This paper forms Part VII of "Physico-chemical Approach to Biopharmaceutical Phenomena" Preceding paper, Part VI: H. Nogami, T. Nagai, and H. Umeyama, *Chem. Pharm. Bull.* (Tokyo), **18**, 328 (1970).
2) A part of this work was presented at the 89th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1969.
3) Location: *Hongo, Tokyo; a*) To whom communications should be directed.
4) C. Tanford, *J. Am. Chem. Soc.*, **84**, 4240 (1962).
5) R.E. Weber and C. Tanford, *J. Am. Chem. Soc.*, **81**, 3255 (1955).
6) A. Yaron, N. Lupu, M. Sela, and A. Berger, *Biochem. Biophys. Acta.*, **69**, 430 (1963).
7) C. Tanford and P.K. De, *J. Biol. Chem.*, **236**, 1711 (1961).
8) K. Hamaguchi and K. Imahori, *J. Biochem.*, **55**, 388 (1964).
9) C. Tanford, P.K. De, and V.G. Taggart, *J. Am. Chem. Soc.*, **82**, 6028 (1960).
10) W. Kauzman, *Advances in Protein Chem.*, **14**, 1 (1959).
11) K. Shinoda and M. Fujihira, *Bull. Chem. Soc. Japan*, **41**, 2612 (1968).

Tryptophan, one of essential amino acids, consists of the hydrophobic and the hydrophilic moieties, possibly offerring itself as a model of protein segment. Therefore, the effects of denaturants on hydrophobic and hydrophilic moieties of tryptophan may be extended to those on hydrophobic and hydrophilic moieties of protein segment as an approach to an understanding of denaturation phenomena of protein. In other words, useful informations for the denaturation phenomena may be given from the experiments with such a substance of low molecular weight as tryptophan, without using a protein. Upon discussion of the effects of denaturants on tryptophan, it is significant to determine the thermodynamic function of the transfer of tryptophan from an aqueous solution to another solution containing the additive, which are easily obtained from the solubilities in both solutions. In this connection, such functions obtained from solubilities were applied by Nozaki and Tanford to comparing of hydrophobicities of various amino acids.[12,13]

In this study, the solubility properties of tryptophan were observed in various solutions of the denaturants for protein reported by Tanford, *et al.*,[7,9,14] and it seemed possible to classify the denaturants (or additives)[15] into three types according to the balance of the effects on hydrophobic and hydrophilic moieties of tryptophan resulting in a change of solubility. In comparison with the existing data,[7,9,14] the same classificantion might be extended to the denaturants according to their effects on the denaturation of β-lactoglobulin, and it was established that the solubility of tryptophan or the denaturation of β-lactoglobulin varies with the effects of additive (or denaturant) on hydrophobic and hydrophilic moieties of molecule, where the decrease in the enthalpy of mixing was considered to be a predominant factor to make the solubility of tryptophan increase or to cause the denaturation of β-lactoglobulin.

Experimental

Materials——L-Tryptophan, MeOH, EtOH, *n*-PrOH, urea, formamide, N,N-dimethylformamide, dioxane, acetone, ethylene glycol, methylcellosolve, butylcarbitol, diethylene glycol, carbitol, and triethylene glycol of the purest grade were obtained commercially.

Procedure of the Determination of Solubility of Tryptophan——Adding the excess of L-tryptophan (about 0.8 g) in 20 ml of solution, the procedure was carried out in the same way as described in the previous paper.[1]

Results and Discussion

Change in Solubility of Tryptophan by Effects of Additive on Hydrophobic and Hydrophilic Moieties of the Solute

As shown in Fig. 1, regarding an identical concentration of 3rd component (additive), the increase in hydrophobicity orginated from introduction of hydrophobic group in the moleculer structure of additive resulted in an increase in solubility of tryptophan in low added concentration, while it resulted in a decrease in solubility in high added concentration. However, this tendency was not remarkable in the high added concentration for butylcarbitol in comparison with carbitol, as might be due to too large an increase in solubility in the low added concentration caused by the introduction of hydrophobic group. A similar phenomenon was observed for the solubility of tryptophan in aqueous MeOH, EtOH, or *n*-PrOH, as is shown in Fig. 2—a and will be discussed later.

Nozaki and Tanford reported that the solubilities of glycine, alanine and leucine in water decreased gradually with addition of ethylene glycol (30%, 60%, and 90%) and also decreased

12) Y. Nozaki and C. Tanford, *J. Biol. Chem.*, **238**, 4074 (1963).
13) Y. Nozaki and C. Tanford, *J. Biol. Chem.*, **240**, 3568 (1965).
14) C. Tanford and C.B. Buckley, *J. Biol. Chem.*, **237**, 1168 (1962)
15) The terms "denaturant," "additive," and "3rd component" are used for convenience of explanation, having no special discrimination.

at an identical concentration of the same additive in the following order: glycine>alanine>leucine.[13] However, as shown in Fig. 1, the solubility of tryptophan increased with addition of ethylene glycol. Therefore, the solubilities of such amino acids in water–ethylene glycol system may depend on the size of hydrophobic moiety of solute molecule, and accordingly the increase in solubility of tryptophan with addition of ethylene glycol may be due to the large hydrophobic moiety of tryptophan. As a possible explanation for the case where a maximum is given on the plot of solubility against added concentration, it is considered that such an additive molecule has more intense effect on the hydrophilic moiety to result in a decrease in solubility in the high added concentration. In this connection, a competitive interaction of two kinds of additives with the hydrophobic or the hydrophilic moiety of tryptophan was observed from the solubility properties of tryptophan in urea–ethanol–water system.[16] Further investigations should be made for this problem.

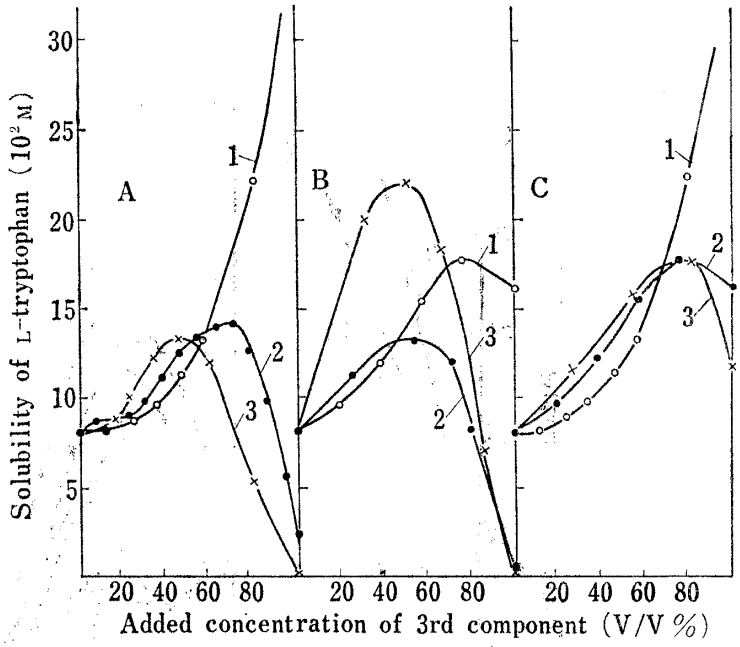

Fig. 1. Effect of 3rd Component on Solubility of L-Tryptophan at 30°

A 1. ethylene glycol 2. methyl cellosolve 3. *n*-PrOH
B 1. diethylene glycol 2. carbitol 3. butyl carbitol
C 1. ethylene glycol 2. diethylene glycol 3. triethylene glycol

Three Types of Additives according to the Balance of Their Effects on Hydrophobic and Hydrophilic Moieties of Tryptophan

Additives having influence on the solubility of tryptophan are classified into three types according to the solutility–added concentration curve, as shown in Fig. 2-a.

Regarding the type I, *i.e.*, urea and formamide, the solubility increases linearly with the addition of them up to 60—70% addition. Considering that the solubilities of glycine and alanine decreased gradually with the addition of urea,[12] the increase in solubility of tryptophan with addition of urea was considered to come from the effect of urea on the hydrophobic moiety of tryptophan, *i.e.*, indole group. The addition of formamide made a maximum solubility of tryptophan, *i.e.*, 36.0×10^{-2}M around 80% addition, then making a little decrease down to 29.3×10^{-2}M of the solubility in 100% formamide.[17] These facts mean that the increase in solubility in this type comes from the predominant effect of additive on the hydrophobic moiety of tryptophan but not on the hydrophilic moiety.

Regarding the type II, *i.e.*, MeOH, EtOH, *n*-PrOH, acetone, dioxane, and N,N-dimethylformamide, the plot of solubility of tryptophan against added concentration has a maximum around 50% addition, and moreover a minimum is found in the case of MeOH, EtOH, or *n*-PrOH around 25% addition. Regarding the increasing part of curve, the effect of additive on hydrophobic moiety of tryptophan may overcome that on hydrophilic moiety to result in the increase in solubility, and regarding the decreasing part of curve, the effect on hydrophilic moiety may overcome that on hydrophobic moiety to result in the decrease in solubility.

16) H. Umeyama, T. Nagai, and H. Nogami, to be published.
17) These values are not shown in Fig. 2-a.

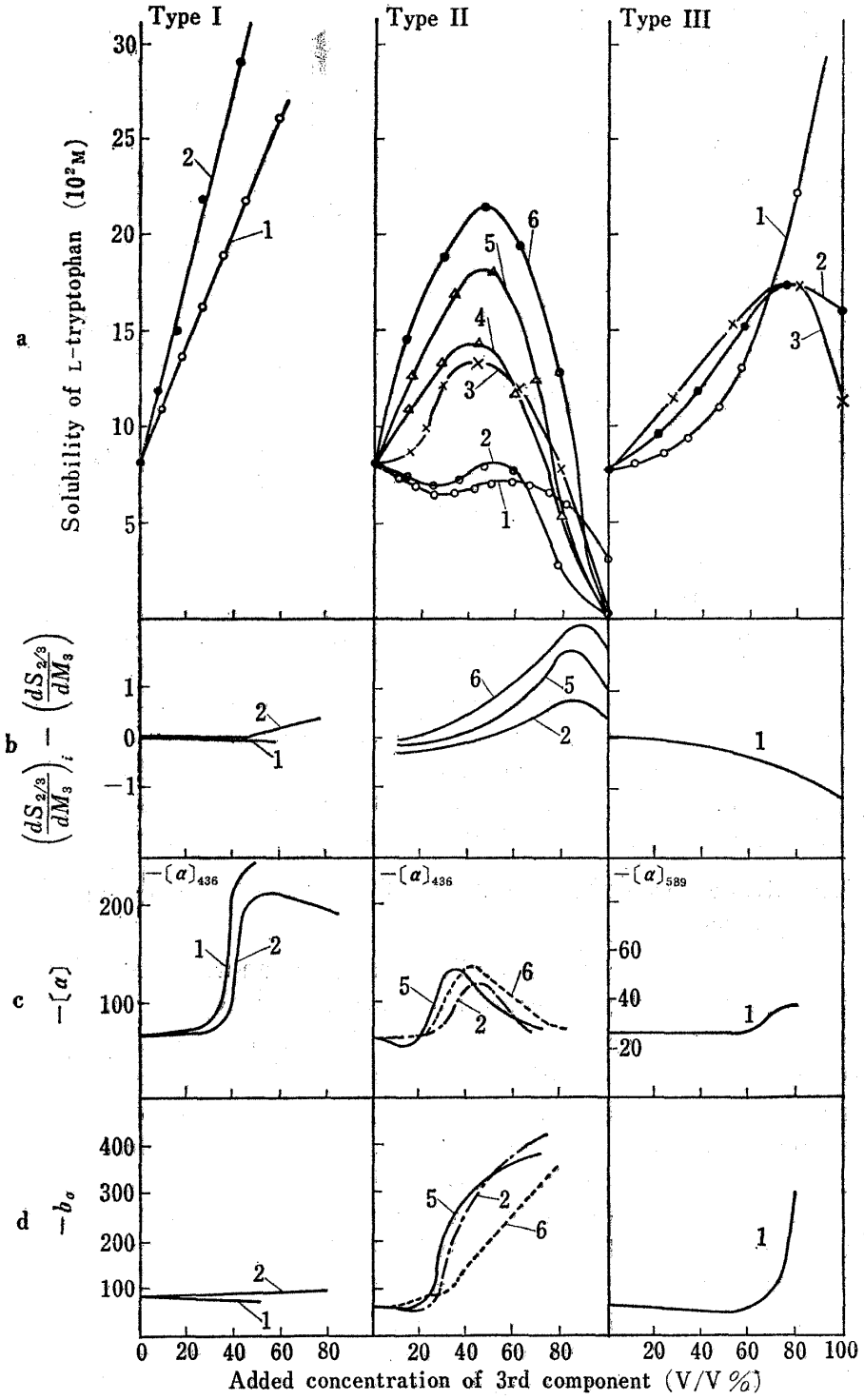

Fig. 2. Effect of Three Types of 3rd Components on Solubility of L-Tryptophan and on Denaturation of β-Lactoglobulin

Type I 1. urea 2. formamide
Type II 1. MeOH 2. EtOH 3. *n*-PrOH 4. acetone 5. dioxane 6. N,N-dimethyl formamide
Type III 1. ethylene glycol 2. diethylene glycol 3. triethylene glycol
a. effect on solubility of L-tryptophan
b. $\left(\frac{dS_{2/3}}{dM_3}\right)_i - \left(\frac{dS_{2/3}}{dM_3}\right)$ obtained from Fig. 2-a $\left(\text{for EtOH in } M_3 < 7\text{M}, \left(\frac{dS_{2/3}}{dM_3}\right) - \left(\frac{dS_{2/3}}{dM_3}\right)_{M_3=7}\right)$
c. Effect on specific rotation, $-[\alpha]$ of β-lactoglobulin reported[7,9,14] (For N,N-dimethyl formamide, $-[\alpha]_{436}$ was estimated from original data of $-[\alpha]_{589}$, comparing $-[\alpha]_{589}$ of dioxane)
d. effect on helix constant, $-b_0$ of β-lactoglobulin reported[7,9,14]

In the cases of MeOH, EtOH, and *n*-PrOH, even in the low added concentration, an effect of additive on hydrophilic moiety may be produced in the lower addition than 25% to result in the decrease in solubility down to the minimum, after which the solubility may increase in the same mechanism as in the cases of acetone, dioxane, and N,N-dimethylformamide.

Regarding the type III, *i.e.*, ethylene glycol, diethylene glycol, and triethylene glycol, an effect on hydrophilic moiety of tryptophan may be produced in the high added concentration, being not so intense. Moreover, the effect on hydrophobic moiety in the low added concentration is considered to be not so intense as types I and II, as shown by the increasing curves of solubility in Fig. 2-a.

Viewing the above three types, it is found that the classification is flexible. For instance, both formamide (type I) and N,N-dimethylformamide (type II) have the respective maxima on the curves of solubility. Here, the differences between both curves are that the former gives the maximum around 80% addition[17] and the latter does aronud 50% addition and that the former shows the higher maximum solubility than the latter. However, as will be discussed later, the classification in those three types is helpful to analyzing of the denaturation data of globular protein.

Relationship between Solubility Properties of Tryptophan and Denaturation Data of β-Lactoglobulin in the Aqueous System Containing the Above Three Types of Additives

Tanford, *et al.*, reported the denaturation of β-lactoglobulin in solutions of various denaturants by determining the optical rotatory dispersion.[7,9,14] Comparing their data of specific rotation, α and dispersion parameter, b_0 with the present solubility properties of tryptophan in the solutions of additives (or denaturants), the denaturants for β-lactoglobulin also can be classified into the same three types as the additives having influence on the solubility of tryptophan, as shown in Figs. 2-c and 2-d. Citing the schematic representation of both the native and the intermediate configurations of protein in Fig. 3,[5] the optical rotatory dispersion data may show that the protein changes with addition of the three types of denaturants as follows:

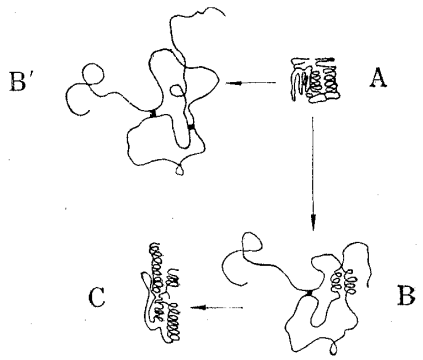

Fig. 3. Schematic Representation of both Native and Intermediate Configurations of Protein[5]

Type I: A (native) ⟶ B′ (unfold)

Type II: A (native) ⟶ B (unfold) ⟶ C (helical)

Type III: A (native) ⟶ B (unfold)

RegardingA⟶B′ and A⟶B, the denaturant may have effect on the hydrophobic moiety of protein to bring into contact with the solvent or to bring into a spreading state. Next, regarding B⟶C, the denaturant may have effect on the hydrophilic moiety of protein to hinder from attaching to the solvent or to bring into a compact state, resulting in a helical configuration.

The data showed that the denaturation of β-lactoglobulin by a type of denaturant corresponded well to the solubility affected by the same type of additive.

In order to indicate the degree of the effect of additive on hydrophilic moiety of tryptophan, the value $\left(\frac{dS_{2/3}}{dM_3}\right)_i - \left(\frac{dS_{2/3}}{dM_3}\right)$ was plotted against the added concentration in Fig. 2-b, where $S_{2/3}$ is the solubility of tryptophan in the solution containing additive, M_3 is the concentration of additive, both in mole unit, and the suffix i means the initial concentration of M_3. Except for EtOH, $i=0$. For EtOH, $i=7$M where a minimum of solubility of tryptophan was given as shown in Fig. 2-a, and $\left(\frac{dS_{2/3}}{dM_3}\right) - \left(\frac{dS_{2/3}}{dM_3}\right)_{M_3=7}$ was plotted in the lower concentration

than $M_3=7$. The increase of such a value indicates the increased effect of additive on hydrophilic moiety. The results in types I and II corresponded well to the denaturation of β-lactoglobulin by the respective types of denaturants. In the case of type III, *i.e.*, ethylene glycol, the value $\left(\frac{dS_{2/3}}{dM_3}\right)_i - \left(\frac{dS_{2/3}}{dM_3}\right)$ was negative and decreased with the concentration of additive, as shown in Fig. 2-b, meaning that the effect on hydrophilic moiety increased with the concentration of additive and that the result corresponded well to the denaturation of β-lactoglobulin by the same denaturant.

It was, therefore, established that the effects of additive (or denaturant) on hydrophobic and hydrophilic moieties of tryptophan, which were observed from solubility properties of tryptophan, might be extended to those on hydrophobic and hydrophilic moieties of such a globular protein as β-lactoglobulin resulting in a denaturation. Accordingly, it is possible to consider the two simultaneous effects of denaturant on the segment in protein molecule resulting in a denaturation, *i.e.*, one on the non-polar side chain (hydrophobic group) and another on the peptide chain (hydrophilic group), and the degree and way of denaturation may depend on the balance of the above two effects in a given concentrtion of denaturant.

Discussions on Solubility Properties of Tryptophan and Denaturation of Globular Protein upon Addition of Third Component based on the Enthalpy of Mixing

In the previous paper,[1] the thermodynamic functions of transfer of tryptophan from aqueous solution to aqueous solution of 3rd component were expressed in the following equations:

$$\Delta H^m_{2/3}/T + (\Delta H^i_{2/3}/T - \Delta S^i_{2/3}) = -R \ln (X_{2/3}/X_{2/0}) \qquad (1)$$

where $\Delta H^m_{2/3}$ is the enthalpy of mixing of solute with solvent upon addition of 3rd component at the temperature T, $\Delta H^i_{2/3}$ the enthalpy ($\Delta S^i_{2/3}$ the entropy) of structural change of the iceberg around solute upon addition of 3rd component, $X_{2/3}$ the molar fraction of solute in the aqueous solution containing 3rd component, and $X_{2/0}$ the molar fraction of solute in water. Then it was considered that the increase in solubility of tryptophan upon addition of 3rd component is due to the predominant decrease of enthalpy of mixing, $\Delta H^m_{2/3}$, depending on the change of the following experimental constant B':

$$B' = (\delta_1 - \delta_2)^2 \qquad (2)$$

where δ_1 and δ_2 are the the solubility parameters of solvent and solute, respectively. Moreover, δ_2 was considered to consist of the solubility parameter of hydrophobic moiety, $\delta_2(a)$ and that of hydrophilic moiety, $\delta_2(b)$, where $\delta_2(a)<\delta_1<\delta_2(b)$ for tryptophan in aqueous solution.

Regarding the type I of additives (denaturants), as was discussed in detail for the case of ureas in the previous paper,[1] the additive may come in contact with the hydrophobic moiety of tryptophan to result in a simultaneous structural change (a kind of destruction) of the iceberg around that moiety, accompanying an increase of its affinity to the solvent, that is, an increase in $\delta_2(a)$ and finally an increase in solubility of tryptophan. For β-lactoglobulin, the denaturant may come in contact with the nonpolar side chain to result in a simultaneous structural change of iceberg, accompanying a decrease of the enthalpy of mixing, that is, an increase in $(X_{2/3}/X_{2/0})$ in equation (1). The increase in $(X_{2/3}/X_{2/0})$ means that the hydrophobic moiety of the protein is more soluble or dispersible in the solution containing 3rd component than in water. This is considered to correspond to the change A→B′ in Fig. 3.

Robison and Jencks reported on the interaction of urea with a hydrophilic polypeptide chain, which was explained from the facts that the solubility of acetyltetra-glycine ethylester increased upon addition of urea and that the increase in solubility of carboxyglycine amide upon addition of urea was larger than that of toluene or benzyl alcohol.[18] However,

18) D.R. Robinson and W.P. Jencks, *J. Am. Chem. Soc.*, **87**, 2462 (1965).

since it was reported that such bondings as C···OHN, CO···HOH, H_2O···HN, CO···Urea and Urea···HN essentially had almost the same stability,[7] the hydrogen bonding in a helix in the hydro phobic moiety may not be broken competitively by urea. Actually, the helix constant did not increase with the addition of urea, as shown in Fig. 2-d. Certain polypeptide chains are included in a hydrophobic moiety avoidably through the formation of folded structure by hydrophobic chains. Therefore, urea may have effect also on such a polypeptide chain to result in an increase in its affinity to the solvent, but this effect may be too weak to change the whole configuration of the hydrophobic moiety where the hydrophobic side chains play a predominant role. Accordingly, the effect of urea on a polypeptide chain can not be a primary factor for the denaturation of globular protein.

Regarding the type II, the increase in solubility of tryptophan with the concentration of additive may depend on the increase in $\delta_2(a)$ by the effect of additive on hydrophobic moiety of tryptophan, but the additional amount of additive in contact with that moiety may get small over about 50% addition because of reaching a saturation, *i.e.*, reaching an equilibrium in structural change of iceberg where $\delta_2(a)$ gets constant. On the other hand, the addition of organic solvent causes a decrease in an affinity of hydrophilic moiety to the solution, accompanying a decrease in δ_1. Therefore, over about 50% addition where a maximum solubility is given, the value of B' in equation (2) increases to result in an increase in enthalpy of mixing, $\Delta H^{m}_{2/3}$, that is, in a decrease in solubility of tryptophan. The above consideration may be extended to the effects of additive (denaturant) of type II on hydrophobic and hydrophilic moieties of β-lactoglobulin resulting in a denaturation, as was explained in the cases of denaturants of type I.

Regarding the type III, an increase in the enthalpy of mixing, $\Delta H^{m}_{2/3}$ by the effect on hydrophilic moiety may be small, while the decrease in $\Delta H^{m}_{2/3}$ by the effect on hydrophobic moiety may be predominant, resulting in the increase in solubility of tryptophan, though the increasing tendency was not so remarkable as in the cases of types I and II. Conclusively, the denaturation of β-lactoglobulin by the denaturant of type III also may be explained by the extension of the consideration made in the cases of types I and II, and it correspond to a weakened one of the denaturation by type II.