BIOPOLYMER SOLUTIONS AND MODEL SYSTEMS UNDER HIGH PRESSURE*

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The evidences are presented that the effects of pressure on proteins are inverted. in the range 2,000~3,000 atm This curious inversion can be illustrated according to the following conclusion derived from the studies of the model systems: pressures below about 3,000 atm favor the formation of hydrogen bonds and the disruption of hydrophobic bonds, and above this pressure these trends are inverse in aqueous solutions. All of these inversion phenomena in the range 1,000~3,000 atm will reflect the change of water structure under high pressure.

Deoxyribonucleic acids (DNA) can not be denatured by compression up to 10,000 atm in contrast with proteins. The facts are discusssed from the view of the differences in conformations between DNA and proteins.

Biopolymer Solutions

Our studies on protein denaturation under high pressure have been performed since 1957. In the present paper the common features of high pressure denaturation of proteins will be summarized. The pressure effect on DNA had never been found until the recent publication of Hedén, Lindahl and Toplins (1963)[1]. After a while we have taken up the pressure denaturation of DNA's from salmon sperm and calf thymus. Pressure up to 10,000 atm could not cause any denaturation of DNA as the result of Hedén *et al.* on DNA of *B. subtilis*. This negative result, however, will be worthy of publishing in comparison with proteins. The studies of the effect of pressure on synthetic biopolymers are important in themselves and for the elucidation of the pressure denaturation of biopolymers. In this communication the effect of pressure on the ionization degree of poly-D-glutamic acid will be presented.

Denaturation of proteins under high pressure[2][3]

Figure 1 shows some typical results of the pressure denaturation of globular proteins. Generally speaking, the denaturation starts at 2,000~6,000 atm and becomes prominent with the increase of pressure, though the pressure under which the denaturation starts depends on temperature, pH, and the ionic strength of the solvent. It has been confirmed that the denatured proteins shown in Fig. 1 do not renature after the release of pressure. In serum albumin, however, the regeneration proceeded very rapidly. The denaturation, checked by the turbidity measurement directly under high pressure, also started at 4,000~5,000 atm (pH 4.8, 0.1 mole acetate buffer)[4].

Figure 2 shows some typical results of the retardation effect of pressure on heat denaturation and

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1) L. G. Hedén, T. Lindahl and I. Toplins, *Acta Chem. Scand.*, **18**, 1150 (1964)
2) K. Suzuki, *Seibutsu-butsuri*, **3**, 4 (1964)
3) C. Suzuki and K. Suzuki, *Tanpakushitsu Kakusan Kouso* (*Protein Nucleic Acid Enzyme*), **11**, 1246 (1966)
4) K. Suzuki, Y. Miyosawa and C. Suzuki, *Arch. Biochem. Biophys.*, **101**, 225 (1963)

the acceleration effect of pressure on the renaturation process of the proteins once denatured by pressure. An important fact that the effect of pressure on proteins is inverted in the range 2,000～3,000 atm is deduced from the results of Figs. 1 and 2.

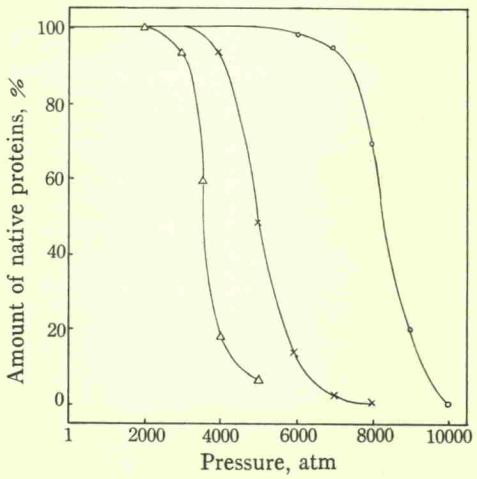

Fig. 1 Effect of pressure on denaturation of proteins

	pH		Conc. (%)	Temp. (°C)
—○—: α-amylase[5]	5.8	(0.05 mole acetate buffer, 10^{-2} mole CH_3COOCa)	3×10^{-4}	20
—×—: γ-globulin[6]	6.0	(0.01 mole phosphate buffer, 0.25 mole NaCl)	0.5	30
—△—: β-lactoglobulin[7]	5.2	(0.1 mole acetate buffer)	1.0	30

Pressure duration: 5 min.

The denaturation was checked from the activity measurement in α-amylase, and from the solubility measurement at isoelectric points in other proteins.

Stability of deoxyribonucleic acids under high pressure[8]

Salmon sperm DNA (Lot. 5028) and calf thymus DNA (Lot. 1513, 1790) were purchased from CalBiochem. and Sigma Co. Ltd., respectively. No change in the optical density of DNA solution at 260 mμ was observed immediately after the release of pressure for DNA solutions (DNA conc. 2×10^{-3} ～4×10^{-2} %, pH 4.8～9.9), which were exposed to 10,000 atm for 60 min at 25～40°C. Expecting the rapid recovery of denaturation, then, formaldehyde was added beforehand to be 1 per cent to prevent the reformation of hydrogen bonds. In this case the difference in the optical density at 260 mμ was not found either between native DNA and pressure treated DNA. These results show that the exposure of DNA to a pressure up to 10,000 atm does not cause denaturation. This fact coinsides with the result of Hedén *et al.*[1] on DNA from *B. subtilis* which was tested with transformation, and is very interesting in comparison with the fact that proteins are denatured by pressure above 2,000～6,000 atm. This must reflect the differences in conformations between DNA and proteins.

5) K. Suzuki and K. Kitamura, *J. Biochem.*, **54**, 214 (1963)
6) K. Suzuki and Y. Miyosawa, *ibid.*, **57**, 116 (1965)
7) K. Suzuki, Y. Miyosawa and E. Miyamoto, *16th Annual Meeting of Chemical Sosiety of Japan*, Tokyo (1963)
8) K. Suzuki and Y. Miyosawa, *The 7th International Congress of Biochemistry*, Tokyo (1967)

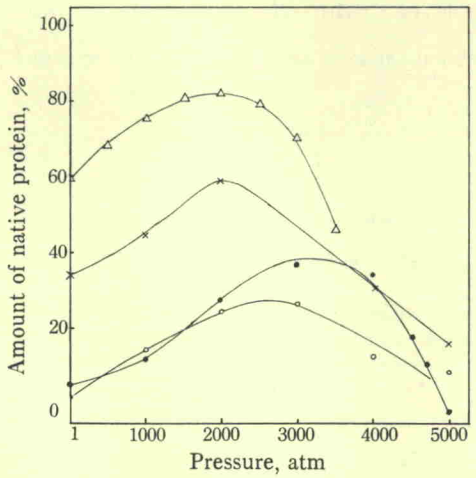

Fig. 2 Effect of pressure on heat denaturation of proteins (—×—, —●—) and effect of recompression on pressure denatured proteins (—△—, —○—)

	Conditions of denaturation	Conditions of renaturation
—×—: γ-globulin[6]	0.5 %, pH 6.0 70°C, 5 min	
—○—: ovalbumin[9]	0.7 %, pH 4.8 70°C, 20 min	
—△—: β-lactoglobulin[7]	1 %, pH 5.2 3,500 atm, 30°C, 5 min	30°C, 60 min
—○—: α-amylase[5]	2×10^{-4} %, 10^{-2} mole CH_3COOCa 10,000 atm, 30°C, 5 min	$2/3\times10^{-4}$ %, pH 7.5 (1/30 mole Tris buffer), 30°C, 60 min

The denaturation was checked from the same methods in Fig. 1.

Ionization degree of poly-D-glutamic acid under high pressure[10]

The sodium salt of poly-D-glutamic acid (PDGA, MW=83,000) was donated from Dr. J. Noguchi, Hokkaido University. By the ionexchange (Amberlite IR-120) the polymer solution of salt type was changed into the solution of acid type. The molar conductivity (Λ) of PDGA in aqueous solution (pH 3.75, conc. 9.30×10^{-3} mole) was measured at 30°C up to 4,500 atm. The increase in Λ is due to the enhanced ionization of the weak electrolyte at high pressure, and the conductivities can be used to derive the change in the degree of ionization, provided that allowance is made for both the effects of pressure and ionic strength on the mobilities of the ions. The degree of dissociation was derived by Hamann's method[11] for simple weak electrolyte.

We assume that ionic conductivities $\Lambda_{\text{polyion}^-}$ and Λ_{H^+} in an ionized solution of ionic strength μ and at the pressure P, will be the same as those in the solutions of NaPDGA and HCl at the same ionic strength and pressure. Further if we assume that NaPDGA, NaCl and HCl are completely ionized, and there are no changes of counterions' concentration by the binding effect of counterions on account of high ion atmosphere around polyion, it follows that

9) K. Suzuki, *This Journal*, **28**, 24 (1958)
10) K. Suzuki and Y. Taniguchi, *Biopolymers*, **6**, 215 (1968)
11) S. D. Hamann, "Physico-Chemical Effects of Pressure", Butterworths Scientific Publications, London (1957)

$$\Lambda_{\text{polyion}^-}+\Lambda_{H^+}=\Lambda_{\text{polyCOONa}}+\Lambda_{HCl}-\Lambda_{NaCl}$$

where all the Λ's are measured at the ionic strength μ and the pressure P. The degree of ionization α is then given by

$$\alpha=\left[\frac{\Lambda_{\text{polyCOOH}}}{\Lambda_{\text{polyion}^-}+\Lambda_{H^+}}\right]_{\mu,P}$$

Some values of α derived by this method are listed in Table 1. It is seen that the extent of ionization of this solution of PDGA increases from about 3 per cent at 1 atm to about 5.3 per cent at 4,500 atm.

Table 1 The molar conductivities Λ's and the degree of ionization α of PDGA in water at 30°C The ionic strength of each solution was 0.01. (The units of Λ's are $\Omega^{-1}\,cm^2\,mole^{-1}$)

P, atm	Λ_{PDGA}	Λ_{NaPDGA}	Λ_{NaCl}	Λ_{HCl}	$\Lambda_{\text{polyion}^-}+\Lambda_{H^+}$	$\alpha\times10^2$
1	8.50	49.10	121.46	355.92	283.56	3.00
900	10.72	52.23	125.37	389.64	316.50	3.39
1,800	12.88	53.29	124.88	398.40	326.81	3.94
2,700	14.66	53.02	122.41	401.58	332.19	4.41
3,600	16.11	52.23	118.33	402.46	336.36	4.83
4,500	17.91	50.99	113.82	400.37	337.54	5.30

Model Systems of Biopolymer Solutions

In order to make clear the above mentioned facts that the effect of pressure on proteins is inverted in the region 2,000～3,000 atm, and the pressure up to 10,000 atm can not cause the denaturation of DNA in contrast with proteins, it will be pertinent to study the effect of pressure on some appropriate model systems related to the bondings which are responsible for maintaining the conformations of proteins and DNA.

The effect of pressure on the low critical solution temperature[12]

2, 6-Dimethylpyridine was used as a sample. This compound is composed of hydrophobic part and hydrophilic part (in which hydrogen bond is capable to be formed) as in other organic compounds which show the low critical solution temperature (LCST). In Fig. 3 the effect of pressure on the LCST of 2, 6-dimethylpyridine—water system is shown. A maximum appears in the figure. The same results have been recently found by Schneider[13] on other systems of the derivatives of pyridine, pyperidine, imine, and nicotine—water. These facts illustrate that the solubility of such a compound into water increases up to 1,000～3,000 atm and then decreases above this pressure.

The effect of pressure on the cloud point[14]

Polyoxyethylene nonylphenyl ether, $C_9H_{19}C_6H_4O(CH_2CH_2O)_{8.2}H$ was used as a sample of nonionic surfactants, which was donated by Daiichi Pharmacentrical Co. Any publication of the effect of

12) K. Suzuki, Y. Miyosawa and M. Tsuchiya, *The 19th Annual Meeting of Chemical Society of Japan*, Tokyo (1966)
13) G. Schneider, *Z. Physik. Chem. (Frankfurt)*, **39**, 187 (1963)
14) K. Suzuki, M. Tsuchiya and T. Kojima, *The 20th Annual Meeting of Chemical Society of Japan*, Tokyo (1967)

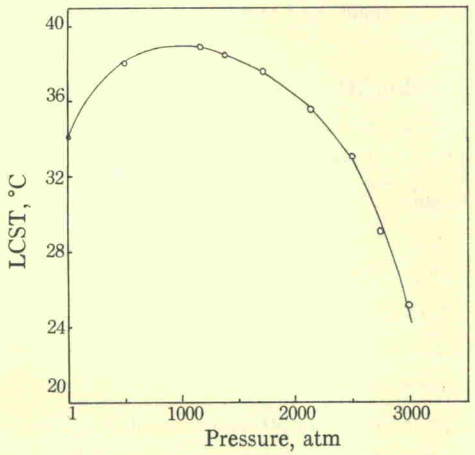

Fig. 3 Effect of pressure on LCST of 2, 6-dimethylpyridine—water
Concentration of 2, 6-dimethylpyridine: 30 wt% (which corresponds to LCST at 1 atm)

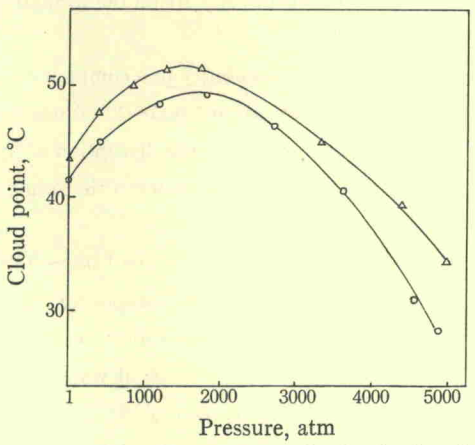

Fig. 4 Effect of pressure on the cloud point of polyoxyethylene nonylphenyl ether ($C_9H_{19}C_6H_4O(CH_2CH_2O)_{8.2}H$ — water
—○—: 0.05 wt% —△—: 10.0 wt%

pressure on the cloud point has never been found. Figure 4 shows the effect of pressure on the cloud point, and is quite similar to Fig. 3. This result also illustrates that there is a maximum at 1,000～2,000 atm in the solubility of the nonionic surfactant into water.

The effect of pressure on UV absorption spectrum of phenol–dioxane system in *n*-hexane[15]

The excellent study of ultraviolet absorption spectrum of phenol-dioxane system in *n*-hexane has been performed at 1 atm by Nagakura[16]. It has been found that the absorption at 270.8 mμ (a-band) and 277.4 mμ (b-band) and that at 273.2 mμ (a′-band) and 279.9 mμ (b′-band) relates to free phenol and hydrogen bonded phenol, respectively. As the concentration of dioxane increases, the optical density at a′- and b′-band ($OD_{a'}$ and $OD_{b'}$) increases and that of a- and b-band (OD_a and OD_b) decreases.

The pressure effect on the absorption spectrum on the same system was examined in the pressure range of 1～5,000 atm and at room temperature. The result shows that the values of $OD_{a'}/OD_a$ and $OD_{b'}/OD_b$ gradually increase with the rise of pressure. This result may be explained to reflect the increase of hydrogen bonded phenol. That is, it is concluded that the increase of pressure favors the formation of hydrogen bonds as in other researches from infrared absorption spectra[17].

Discussion

The volume changes in the transfer of hydrocarbons from nonpolar solvents to water are known to be in the order of -20 ml/mole[18]. This means that the application of pressure favors the disruption of hydrophobic bonds. On the other hand, it is confirmed from the above experimental results of UV absorption spectrum that pressure favors the formation of hydrogen bonds. That is, it should be men-

15) K. Suzuki and M. Tsuchiya, *Preprint of the 9th Symposium on High Pressure*, p. 95 (1967)
16) S. Nagakura, *Nippon Kagaku Zasshi* (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **74**, 87 (1953)
17) E. Fishman and H. D. Drickamer, *J. Chem. Phys.*, **24**, 548 (1956): J. Osugi and Y. Kitamura, *This Journal*, **35**, 25 (1965)
18) W. Kauzmann, *Advances in Protein Chem.*, **14**, 1 (1959)

tioned that the effects of pressure on the formation of hydrogen bonds and hydrophobic bonds are quite opposite each other.

The fact that the solubility of a compound which has hydrophobic group and hydrophilic group in one molecule increases up to 1,000～3,000 atm as shown in Figs. 3 and 4 with the results of Schneider[13] will be explained as follows : the disruption of hydrophobic bonds among the solute molecules and the formation of hydrogen bonds between the solute and the solvent are enhanced with the increase of pressure.

The decrease of solubility above 1,000～3,000 atm in Figs. 3 and 4w ith the results of Schneider[13] will be plausibly explained by supposing the formation of hydrophobic bonds and the disruption of hydrogen bonds above this pressure, respectively. All the inversion phenomena in the range 1,000 ～3,000 atm will reflect the change of water structure under high pressure.

This conclusion is favorable to explain the inversion phenomenon of pressure on proteins. That is, the pressure above 1,000～3,000 atm causes denaturation (by disrupting hydrogen bonds maintaining the conformation of native proteins), and a strong aggregate of proteins (caused by the formation of hydrophobic bonds among denatured proteins). On the other hand, the pressure below 1,000～3,000 atm retards the heat denaturation of proteins (caused by the rupture of hydrogen bonds), and dissolves the hydrophobic bonded aggregates (formed at high pressure above 1,000～3,000 atm).

The reason why DNA can not be denatured by pressure in contrast with proteins will be discussed, though we can not discuss in detail at present. There are significant differences in the formation of hydrogen bonds which are responsible for the helical structure of proteins and for the double helical structure of DNA. Therefore, some differences may be assumed in the hardness of disruption of hydrogen bonds between proteins and DNA. Perhaps more profound differences are expected in the dissociable groups between DNA and proteins. Proteins are weak amphoteric electrolytes, and some undissociated groups may be presented in the native state. While in DNA there is only phosphate group as dissociable group, and phosphate group is perfectly dissociated in the usual pH range.

Pressure favors the ionization process as shown in the present result of poly-D-glutamic acid. Therefore, some undissociated groups in proteins may be ionized by pressure, and the repulsion force to weaken the protein structure occurs between ionized groups. On the other hand, in DNA such a pressure effect does not occur. After all, it will be explained that DNA is not denatured by a pressure up to 10,000 atm.

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