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INACTIVATION OF ENZYMES UNDER HIGH PRESSURE
II INACTIVATION OF BACTERIAL AL-PROTEINASE AND β-AMYLASE OF BARLEY UNDER HIGH PRESSURE

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Inactivations of al-proteinase (EC 3. 4. 4. 16) of *B. substilis* and of β-amylase (EC 3. 2. 1. 2) of barley under pressures up to 10,000 kg/cm², and 7,000 kg/cm², respectively, have been investigated.

The inactivations of both the enzymes by heat are retarded by applications of pressure up to certain magnitudes (ca. 2,000 kg/cm²). Further increase of pressure, however, strongly facilitates the inactivations and very rapid inactivations take place even at room temperature by the application of pressures above certain magnitudes. The inactivations are of first order with respects to enzyme concentrations.

From the data of these enzymes together with the data of α-amylases hitherto obtained, thermodynamic quantities concerning the rate processes were calculated and the general scheme of the inactivation of enzymes under high pressure was discussed.

Introduction

For the purpose of our investigations to clarify the nature of the inactivation of enzymes under high pressure, detailed informations concerning the kinetics of the inactivation of various enzyme species should be desirable.

The inactivations of α-amylases (EC 3. 2. 1. 1) from *B. subtilis*[1] and from saliver[2] under high pressure have been previously reported. The inactivations of trypsin[3], chymotrypsin[4] and Taka-amylase-A[5] under high pressure have also been investigated by Miyagawa and Suzuki.

In this report, the inactivations of alkaline proteinase (al-proteinase) of *B. subtilis* (EC 3. 4. 4. 16, subtilo peptidase-A) and of β-amylase of barley (EC 3. 2. 1. 2, α-1.4 glucan malto hydrolase) under high pressure will be discussed and the general inactivation scheme of enzymes under high pressure will be argued.

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1) K. Kitamura and K. Suzuki. *J. Biochem. (Japan)*, **54**, 214 (1963)
2) K. Kitamura, *This Journal*. **35**, 44 (1965)
3) K. Miyagawa and K. Suzuki, *ibid.*, **32**, 43 (1962)
4) K. Miyagawa and K. Suzuki, *ibid.*, **32**, 51 (1962)
5) K. Miyagawa and K. Suzuki, *Arch. Biochem. Biophys.*, **105**, 297 (1964); **106**, 497 (1964)

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Experimentals

Enzymes : Bacterial al-proteinase was supplied from Nagase & Co., Ltd., as a highly purified specimen, extracted from *B. subtilis var. Biotecus*, recrystallized three times and then lyophilized.

The crude sample of β-amylase of barley was obtained from Nutritional Biochemical Co., Ltd., U. S. A. This sample was assumed as enzymatically pure, without contamination of α-amylase, and was thought to be sufficient for the purpose of our experiments.

The pH of al-proteinase test solution was adjusted with phosphate buffer (finally mole/15). The proteinase activity was assayed colorimetrically according to Hagiwara's method[6] (a modification of Folin's method[7]).

The test solution of β-amylase was prepared by dissolving the enzyme in mole/5 acetate-acetic acid buffer of pH=4.8 ; the experiments were carried out at the constant pH. The amylase activity was measured also colorimetrically according to Noelting's method[8] (a modification of Sumner's[9]). A Hitachi EPU-2 B Spectro-Photometer was used for the optical measurements.

Apparatus and procedures : The high pressure apparatus was the same as described previously[10]. The high pressure was generated in a high pressure vessel of thick walled piston cylinder type with a water jacket for temperature control. The enzyme solution prepared was stored in an ice-box before use to prevent the natural inactivation. For each run, about 3 m*l* of the enzyme solution was sealed in a sack of polyethylene and dipped into the pressure transmitting medium (water in most cases) in the pressure vessel. The temperature of the high pressure vessel was regulated by circulation of thermostatted water through the jacket around the vessel with accuracy of 0.1°.

It took at least 2 minutes to raise the pressure up to several thousands atmospheres after a test sample was charged in the vessel. It took also 1 minute at least to take out the sample from the vessel and to release the pressure. The inactivation during these periods of the transient pressure was very small for cases at lower temperatures. It becomes serious, however, at relatively higher temperatures : the rate of inactivation is the larger at the lower pressure. Not only the compression time but the times of the transient pressures had to be kept constant for the estimation of the change during the transient periods, *i. e.* 3 minutes before the compression and 2 minutes after the compression, respectively.

Results

Inactivation of β-amylase of barley : The enzymatic activity of β-amylase of barley diminishes or disappears irreversibly when the enzyme solution is exposed to hydrostatic pressures of several thousands atmospheres at room temperature. The enzyme is also inactivated when the enzyme solution is

6) B. Hagihara, *Ann. Rep. Fac. Sci. Osaka Univ.*, **2**, 35 (1954)
7) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927)
8) G. Noelting and P. Bernfeld, *Helv. Chim. Acta*, **31**, 286 (1948)
9) J. B. Sumner, *J. Biol. Chem.*, **62**, 287 (1925)
10) K. Suzuki, *This Journal*, **28**, 24 (1958)

heated above about 50°C. The inactivation by heat is, however, retarded by applications of pressure up to about 2,000 kg/cm². Fig. 1 shows the time courses of inactivation. The linearness of semi-logarithmic plots of activity retention against time shows that the inactivation by pressure as well as that by heat is of first order with respect to the enzyme concentration.

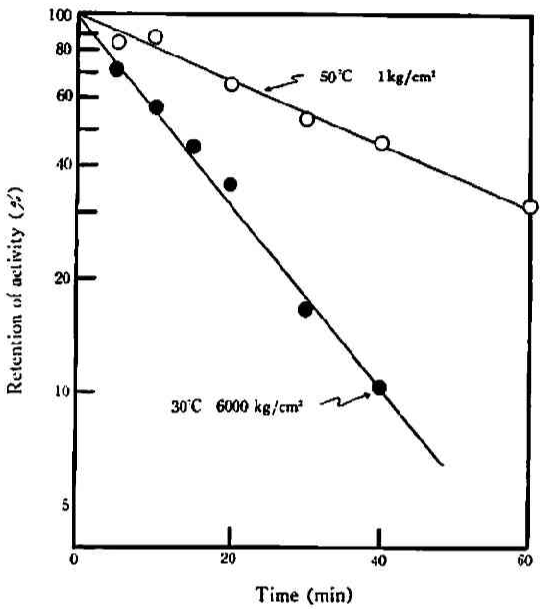

Fig. 1 Time course of the inactivation of β-amylase of barley at pH 4.8

For the cases where the inactivation at atmospheric pressure is very slow and negligible, the first order rate constant k' of the inactivation is calculated simply from the retention of activity A/A_0 after a compression time t, by the formula :

$$k' = -1/t \ln (A/A_0).$$

where A and A_0 are the activities of enzyme in arbitrary scale after and before the compression.

At relatively high pressures, the inactivation during the transient periods when the pressure is raised or released becomes serious, since the rate of inactivation is larger at a lower pressure. The rate constant then has to be calculated considering the inactivations during the transient periods by the equation :

$$k' = -1/t\{\ln(A/A_0)+k'_0 t_0\}.$$

where k'_0 is the mean rate constant during the transient period t_0. The value of $k'_0 t_0$ is given experimentally by the relation :

$$k'_0 t_0 = -\ln(A/A_0)_{t=0}.$$

Fig. 2 shows the relations between log k' and pressure P at temperatures between 10° and 50°C. The graph slopes are positive and decrease with increasing temperature at the high pressure range above 3,000 kg/cm², while the slope is negative at pressures below 2,000 kg/cm² corresponding the retardation of the heat inactivation by pressure.

Fig. 3 shows the Arrhenius plots of the rate of the inactivation at pressures up to 7,000 kg/cm².

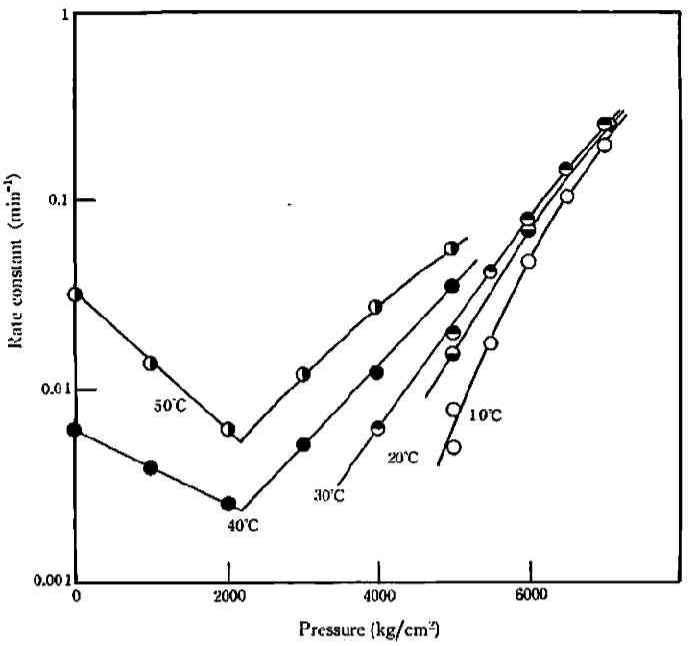

Fig. 2 Relations between log k' and pressure
(β-amylase of barley at pH 4.8)

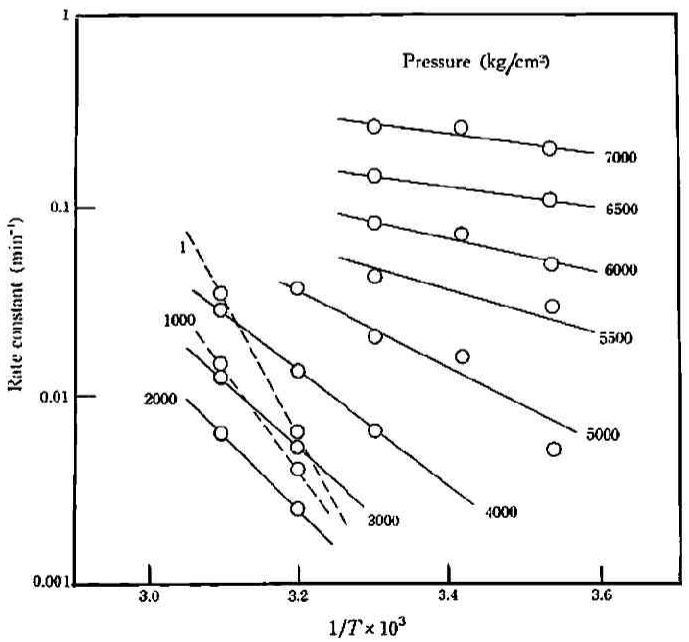

Fig. 3 Arrhenius plot for the inactivation
(β-amylase of barley at pH 4.8)

The temperature coefficient of the rate is positive in whole the experimental range, and decreases

with increasing pressure, correlating to the decrease of the pressure coefficient with increasing temperature as shown in Fig. 2.

Inactivation of bacterial al-proteinase : Although bacterial al-proteinase is very stable against pressure at pH 7.8, the enzyme is inactivated under a pressure of 10,000 kg/cm² irreversibly, following first order kinetics with respect to the enzyme concentration, as shown in Fig. 4. It should be mentioned

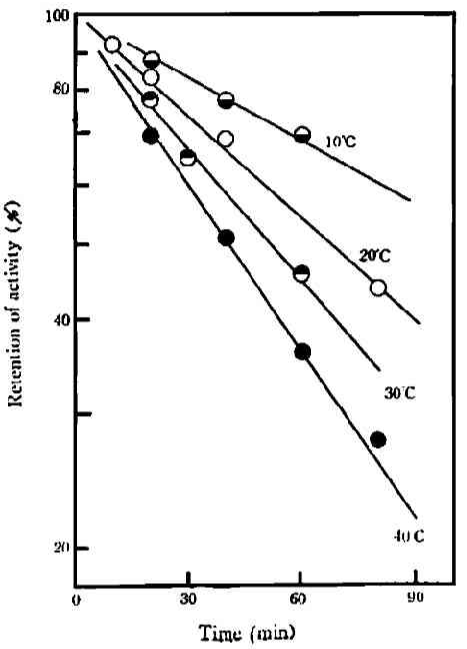

Fig. 4 Time courses of the inactivation of bacterial al-proteinase at pH 7.8, 10,000 kg/cm²

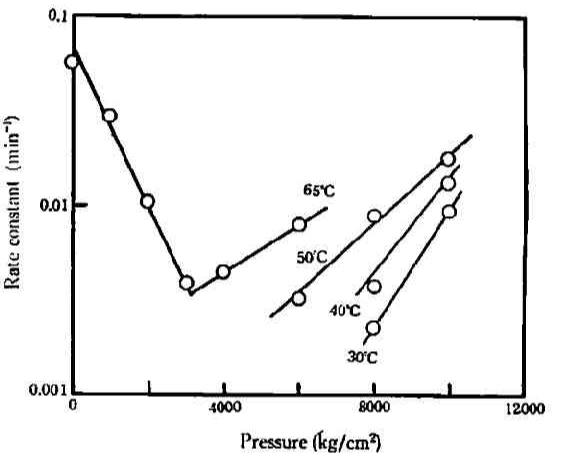

Fig. 5 Effects of pressure on the rate of inactivation (bacterial al-proteinase at pH 7.8)

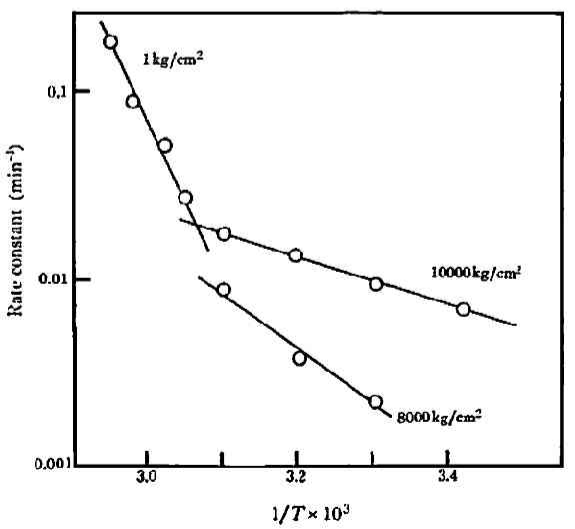

Fig. 6 Influences of temperature on the inactivations by heat and by pressure (bacterial al-proteinase at pH 7.8)

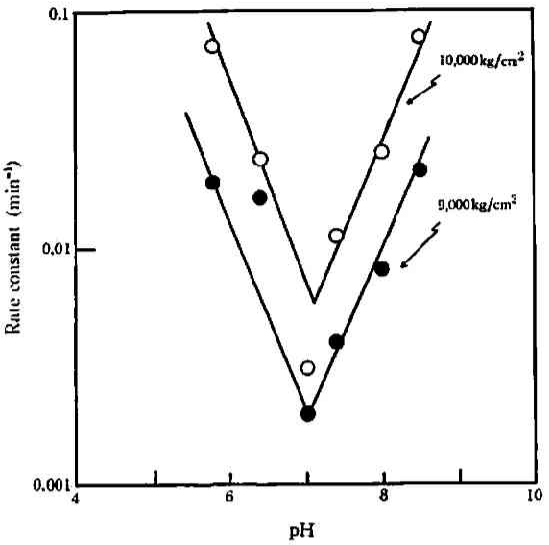

Fig. 7 The effect of pH on the rate of inactivation
(bacterial al-proteinase at 20°C)

that water (accordingly the enzyme solution) is in solid ice-VI state, under the pressure at lower temperatures *e. g.* 20°C or the lower. However, the inactivation occurs regardless of the phase states following the first order law, without abrupt change in the rate constant or in the slope of the Arrhenius plot of the rate constant at the transition point as shown in Fig. 6.

The rate of inactivation is sensitive to pressure as shown in Fig. 5, sensitive to temperature as in Fig. 6 and also sensitive to pH of the media as in Fig. 7. As clearly seen in these figures, the general trends of influences of these variables are similar to those of other enzymes. The rate of inactivation increases with increasing pressure at the high pressure range above 4,000 kg/cm², while the inactivation by heat at atmospheric pressure is retarded by pressures up to 3,000 kg/cm². The rate increases also with an increase in temperature within the whole experimental range. But, the temperature coefficient and the Arrhenius parameter are decreased with increasing pressure. The enzyme is the most stable against pressure at pH 7 ; either lowering or raising the pH from 7 increases the rate. The rate is apparently proportional to hydrogen ion concentration at the acidic side and proportional to hydroxyl ion concentration at the basic side. (The proportionalities are apparent because the values of pH shown in the figure are those at atmospheric pressure, and those at high pressures are not known.)

Discussions

The inactivations of four kinds of enzymes under high pressure have been investigated in this series; two α-amylases previously, β-amylase of barley and bacterial al-proteinase presently. It has been found that these enzymes are inactivated following similar patterns :

(1) The enzymes are inactivated at room temperature (10～40°C) under pressures between 4,000 and 10,000 kg/cm².

(2) The inactivation by heat is retarded by pressures up to some 2,000～4,000 kg/cm², on the

other hand.

(3) The inactivation follows first order kinetic law with respect to the enzyme concentration.

It has been known that the inactivations of trypsin[3], chymotrypsin[4] and Taka-amylase-A[5] also follow these patterns. Moreover, these features are common with those of denaturation of some proteins. The inactivation of enzymes under pressure may be, thus, due to the denaturation of enzyme proteins by common denaturation schemes. It has been, in fact, confirmed that the rates of denaturation and of inactivation of bacterial α-amylase under a high pressure are the same[1].

The difference between the effects of pressure at the higher pressure range (1) and at the lower pressure range (2) seems to indicate the different schemes of inactivation.

The difference is also shown more evidently in the thermodynamic quantities concerning the rate

Table 1 Thermodynamic quantities of the denaturation

	Enzyme or Protein	T °C	P kg/cm²	pH	$\Delta G^{\neq}$ kcal	$\Delta H^{\neq}$ kcal	$\Delta V^{\neq}$ c.c.	$\Delta S^{\neq}$ cal/°C	$\Delta E^{\neq}$ kcal.	$P\Delta V^{\neq}$ kcal	$T\Delta S^{\neq}$ kcal
Denaturation by pressure	Bacterial α amylase[1]	20	7500	5.8	22.0	21.4	−39	−2	28.3	−6.9	−0.6
	Salivery α amylase[2]	30	7500	6.9	22	8.2	−32	−44	13.8	−5.6	−13.8
	β-amylase of barley	30	7000	4.8	21.1	1.7	−33	−64	7.1	−5.4	−19.4
	Bacterial al-proteinase	30	10000	7.8	23.0	5.3	−36	−57	13.8	−8.5	−17.3
	Ovalbumin (hen)[18]	10	4500	4.8	20	−25	−92	−160	−8	−17	−45
	Hemoglobin (bovine)[18]	10	6000	6.8	20	−29	−99	−173	−5	−24.4	−49
by heat	β-amylase of barley	50	0	4.8	23.8	33.4	+23	+30	33.4	0	+9.6
	Bacterial al-proteinase	65	0	7.5	24.6	36.9	+26	+36	36.9	0	+12.3
	Ovalbumin[18]	70	0	4.8	24	102	+12	+220	102	0	+7.8

processes. Table 1 shows the thermodynamic quantities of the inactivations of four enzymes together with those of the denaturations of two proteins: activation enthalpies $\Delta H^{\neq}$, activation volumes $\Delta V^{\neq}$, activation free energies $\Delta G^{\neq}$, activation internal energies $\Delta E^{\neq}$, and activation entropies. $\Delta S^{\neq}$. These quantities were calculated according to the transition state theory of Eyring[11], by the equations:

$$\Delta G^{\neq}=RT \ln (\mathbf{k}\, T/k'\mathrm{h})$$
$$\Delta H^{\neq}=-d \ln k'/d\,(1/T)-RT$$
$$\Delta V^{\neq}=-d \ln k'/dP$$
$$\Delta S^{\neq}=(\Delta H^{\neq}-\Delta G^{\neq})/T$$
$$\Delta E^{\neq}=\Delta H^{\neq}-P\Delta V^{\neq},$$

where R, $\mathbf{k}$ and h are the gas constant, the Boltzmann constant and the Planck constant, respectively.

The thermodynamic features of the transition state of the pressure denaturation and of the heat denaturation are summerized as follows:

(4) The values of $\Delta H^{\neq}$ of the pressure denaturation are generally lower than those of the heat denaturation; both the values decrease with increasing pressure.

(5) The values of $\Delta V^{\neq}$ at the higher pressure range are negative while those at the lower pressure

11) S. Glasstone, K. J. Laidler and H. Eyring, "*The Theory of Rate Processes*", p. 400, Mc Graw-Hill (1941)

range are positive. The negative activation volumes at high pressures become more negative with decreasing temperature.

(6) The values of $\Delta S^{\ddagger}$ are negative at the higher pressure range, while the values at the lower pressure range are positive; while the values at the lower pressure range are positive; the values of $\Delta S^{\ddagger}$ decrease with increasing pressure as a whole.

Table 2 Effects of pH on the rates of the inactivation of proteins and the denaturation of enzymes

Enzymes and Proteins (denaturants)	pH range	$\frac{d\log k'}{d\log[H^+]}$
bacterial al-proteinase (by pressure)[11]	4～ 8	1
salivary α-amylase (〃)[12]	4～10	1
β amylase of barley (〃)	4～ 8	$\frac{1}{2}$
bacterial al-proteinase (〃)	<7	1
〃	>7	-1
ovalbumin (〃)[12]	5～ 7	$\frac{1}{2}$
pepsin (by alkali)[13]	—	-5
hemoglobin (by acid)[14]	—	2
ovalbumin (by acid)[15]	—	2
〃 (by alkali)[16]	—	-4

It is well known that the rates of denaturations by heat, by acids and by alkali are greatly affected by the pH of their media. The rate of pressure denaturation of proteins is also affected by the pH, considerbly, as shown in Table 2 As well as the cases of the denaturations by heat and by acid, the rate of the denaturation by pressure is related to the pH by the relation:

$$k'=k'_{.}[H^+]^n,$$

or

$$d[\log k']/d[\mathrm{pH}]=-n,$$

where k' and n are the constants and independent of pH, respectively. It seems of interest that the values of n for cases of the denaturation by pressure are either 1, 1/2 or -1.

The inactivation scheme to be postulated should be what is capable, of explaining these facts. The denaturation of proteins by heat has been of special interest for many physico-chemists because of extraordinally large activation energies: the transition state from the native state of a protein to the denatured state has been assumed to be nearly the intermediate between the two states. The denaturation of proteins which is a intra-molecular change from a folded helical structure to an unfolded random coiled structure, accompanies splittings of a number of intramolecular hydrogen bonds. The transition state should be then a state with halfly loosened structure, with larger volume and entropy from the initial native state. Applications of pressure may therefore obstruct this volume-increasing process to

12) C. Suzuki, K. Suzuki, K. Kitamura and J. Osugi, *This Journal*, **32**, 37 (1963)
13) V. K. La Mer, *Science*, **86**, 614 (1937)
14) H. Neurath et al., *Chem. Revs.*, **34**, 157 (1944)
15) R. J. Gibbs et al., *Arch. Biochem. Biophys.*, **35**, 216 (1952)
16) R. K. Cannan, *Chem. Revs.*, **30**, 395 (1942)

retard the denaturation.

However, the scheme of the denaturation at the higher pressure range or of the denaturation by high pressure should be far differ from this; the transition state to the pressure denatured state should enhance the decreases in volume and in entropy.

Several speculations on the scheme of pressure inactivation and pressure denaturation have been presented, previously. Eyring and Johnson[17] have ascribed the denaturing effect of high pressure to the changes in the structure of water under high pressure. Suzuki[18] has discussed the denaturation schemes of ovalbumin and of hemoglobin by high pressure and suggested that water under high pressure acts as a strong denaturing reagent as urea or guanidine acts on proteins. Urea and guanidine are thought to act as denaturing reagents by combining with proteins and rupturing the hydrogen bonds between peptide-linkages in the folded helical structures of the native proteins. Water itself is supposed to have the action similar to those of urea or guanidine on proteins, though it may be so weak as to cause the denaturations under ordinary conditions[19].

However, the hydration process as well as the processes of urea addition to proteins, should be a volume decreasing process, so that it may be favoured by increasing pressure. Thus, it seems reasonable that water under high pressure acts as a strong denaturant as urea or guanidine.

This scheme seems to explain rather well the unusual thermodynamic quantities of the high pressure inactivation and the high pressure denaturation in Table 1. The process of hydration should be accompanied by the decreases in entropy of the solution systems as well as the decreases in volumes.

The analogy between the pressure denaturation and the urea denaturation, however, should not be too much stressed, since the effect of pressure on enzyme solutions may be too complicated to be explained with a simple analogy. There are also many differences between the two. For instance, urea denatured proteins are generally dissolved in the denaturing solvent, while pressure denatured proteins are coagulated and separated from the solvent water under pressures. Moreover, pepsin which is very stable in a urea solution, is rapidly inactivated under high pressure.

The rate of the inactivations and the denaturations under high pressure should be also affected by the various factors, such as the electrostatic force between polar groupes in proteins, solvent water and free ions. Some ionic mechanism may co-operate with the hydration to denature proteins, as indicated by the influences of pH on the rate. It may be probable that some ionizable groups in the protein molecule, such as histidine residue, become ionized under pressures and exert electrostatic repulsion forces or attraction forces against the neighbouring ions, weaken some strategically important bonds which maintain the unique conformation of the native protein, and favor the denaturation. It is well known that the ionization constants of weak electrolytes, including water, generally are increased with an increase in pressure, since ionizations are accompanied with the volume decreases of the solution systems.

In any way, the inactivations and denaturations by high pressure may be due to the changes in the properties both of the solvent and the solutes, enzymes and proteins. The changes in the ionization

17) F. H. Johnson, H. Eyring and M. J. Polissar, "*The Kinetic Basis of Molecular Biology*", p. 303, McGraw-Hill (1941)

18) K. Suzuki, *This Journal*, 29, 91 (1959)

19) W. Kauzmann, "*A Symposium on the Mechanism of Enzyme Action* (1954)", p. 70

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states and in the electrostatic forces may have some important roles.

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