biguous, and also the "hydrophobicity" or organicity/inorganicity value[6] of the drugs used in this study is not clear. The pK_a values of nicotinic acid and isonicotinic acid are 4.85 and 4.96,[7] respectively, and more than 91% of these drugs exists in the dissociated state at pH 6.0 and 7.0. However, the pK_a of nicotinamide is 3.35,[7] and it exists almost entirely in the undissociated state at pH 6.0 and 7.0. The pK_{a_1} of nicotine is 6.16.[8] Further work is necessary on the relation between adsorbability and pK_a.

Nicotine is very poisonous and could not be used in these experiments, but from the results obtained in this study, the amount of nicotine adsorbed by HAP might be small because of its larger molecular weight and solubility in water.

Acknowledgement The authors are grateful to Mr. Hiromi Tada for his assistance in the experimental work.

References and Notes

1) This paper forms Part XXXVII of "Physico-chemical Approach to Biopharmaceutical Phenomena." The preceding paper, Part XXXVI: M. Hata, N. Nambu, and T. Nagai, *Chem. Pharm. Bull.*, **29**, 1151 (1981).
2) This work was presented at the 100th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1980.
3) M. Kresak, E.C. Moreno, R.T. Zahradnik, and D.I. Hay, *J. Colloid and Interface Sci.*, **59**, 283 (1977).
4) H. Nogami, T. Nagai, and N. Nambu, *Chem. Pharm. Bull.*, **18**, 1643 (1970).
5) I. Abe, K. Hayashi, M. Kitagawa, and T. Urahata, *Bull. Chem. Soc. Jpn.*, **53**, 1199 (1980).
6) A. Fujita, *Pharm. Bull.*, **2**, 163 (1954).
7) D.W. Newton and R.B. Kluza, *Drug Intell. and Clin. Pharm.*, **12**, 546 (1978).
8) "The Merck Index," 9th ed., Merck & Co., Inc., Rahway, U.S.A., 1976.

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Kinetics of Digestive Enzyme Stability in the Solid State. II.[1] Quantitative Prediction of Enzyme Inactivation

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The procedure for predicting digestive enzyme stability in the solid state was further investigated by utilizing the Weibull distribution function, which needs two mathematically meaningful parameters, m and k. These parameters could be estimated by graphic calculation. If the parameter m is independent of temperature, the Arrhenius plots of $(1/m)\ln k$ *versus* $1/T$ are linear since $k^{1/m}$ is proportional to the inactivation rate constant. The parameter k could be estimated by extrapolating to the desired temperature in the Arrhenius plots and the activation energy could be determined from the slope of this line. These parameters, m and k, made it possible to predict the inactivation ratio of enzymes in the solid state. By comparing the predicted value of the inactivation ratio with the observed value under controlled conditions, it was shown that the proposed method is accurate and useful for studies on enzyme stability in the solid state.

Keywords——Weibull probability paper; digestive enzyme; predicition of stability in solid state; Arrhenius plots; stress test

Many reports on the stability of solid drugs have been published,[2–5] and the degradation ratio of solid drugs during storage could be predicted kinetically by means of the stress test,

providing very important information for estimation of the effective period, for selection of the materials and for determination of suitable preparative conditions, *etc.* Such a kinetic method, however, cannot be adapted to studies on enzyme stability because the inactivation process of enzymes is generally complex. Most enzymes are less stable than various synthetic drugs, but the effective lives of enzymes have not been defined yet.

In the previous paper,[6] we reported that the inactivation process of enzymes in the solid state can be described by a linear regression line on Weibull probability paper. The present paper will deal with quantitative prediction of enzyme stability in the solid state.

Theory

In the most general case of enzymes, no suitable mathematical expression to describe the entire curve of inactivation in terms of meaningful parameters is available.

In the previous paper,[6] we reported that plotting of the inactivation ratios of enzymes on the Weibull probability paper yielded a linear regression line, which is expressed in the following equation;

$$\ln \ln(1/1-\alpha) = \ln k + m \ln t \qquad \text{(Eq. 1)}$$

where ln stands for log with base e, α is the cumulative inactivation ratio during storage, and t, m and k are parameters. Parameters m and k can be easily estimated by simple graphic calculation as described in the previous paper.[6]

The practical application of these parameters to the prediction of enzyme stability in the solid state is similar to that described by Okusa.[7]

The relationship between the parameters estimated from the Weibull probability paper and those estimated from chemical kinetics is as follows; (1) m is the parameter corresponding to the reaction mechanism and (2) $k^{1/m}$ is proportional to the reaction rate constant. Therefore, even though the reaction rate constant is unknown, the activation energy (E) can be estimated from Eq. 2.

$$k^{1/m} = \left(\frac{Z}{d}\right) \exp\left(\frac{-E}{RT}\right) \qquad \text{(Eq. 2)}$$

where d is $\exp A_0$, Z is the frequency constant, $\boldsymbol{R}$ is the gas constant and T is absolute temperature. If the m value is independent of temperature, parallel lines on the Weibull probability paper are obtained for each temperature, but if the m value is dependent upon temperature, no such simple relation is seen, as pointed out by Murty *et al.*[8] For the prediction of enzyme stability, k values for a temperature range having the same m value will be adapted for equation 2. By extrapolating the linear regression line, $(1/m)\ln k$ versus $1/T$, $(1/m)\ln k$ was estimated from the slope of the Arrhenius plots, the activation energy (E) could be determined.

Materials and Methods

Enzymes——The enzymes were microbial lipase from *Aspergillus genus* (marketed as Lipase AP, Amano Pharm. Co., Japan), *Rhizopus sp. NR 400* (marketed as Lipase Saiken, Osaka Saiken Co., Japan) and *Candida cylindracea nov. sp.* (marketed as Lipase MY, Meito Sangyo Co., Japan), microbial α-amylase from *Aspergillus oryzae* (marketed as Biodiastase 1000, Amano Pharm. Co., Japan), pancreatin from porcine pancreas (Amano Pharm. Co., Japan) and diastase from malt (Toyo Jozo Co., Japan).

Stability Test——The stress test was carried out by the procedure described in the previous paper[6] and stability tests during storage were performed by placing each enzyme in a sealed ampule in thermostated water baths at 25°C and 30°C.

Assay Procedure for Enzyme Activity——The activities of lipase and α-amylase were determined according to the method described earlier.[9,10] The remaining activity was expressed as a relative ratio with respect to the enzyme stored in a refrigerator.

Results

Figure 1 shows the Arrhenius plots as a function of $(1/m)\ln k$. Parameters, m and k, at each temperature were estimated by graphic calculation on the Weibull probability paper by the procedure described in the previous paper.[6] The range of temperature within which the Arrhenius plots were linear differed, as shown in Fig. 1. The plots of *Rhizopus* lipase were linear from 45 to 60 °C, but those of pancreatic lipase and α-amylase were only linear below 55 °C, indicating that an appropriate temperature must be selected for the stress test.

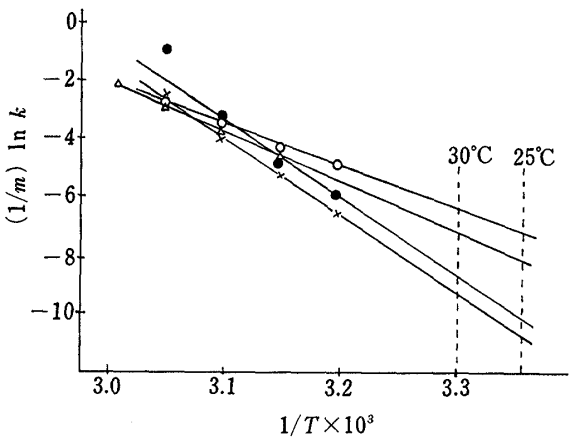

Fig. 1. Arrhenius Plots as a Function of $(1/m)\ln k$ *versus* $1/T$

—●—: pancreatic α-amylase (water content, 7.8%),
—○—: *Aspergillus* α-amylase (water content, 8.6%),
—×—: pancreatic lipase (water content, 7.9%),
—△—: *Rhizopus* lipase (water content, 8.5%).

Prediction of the inactivation ratio at a given temperature may be possible by extrapolating the Arrhenius plots to estimate $(1/m)\ln k$. Under controlled conditions where one parameter, m, is independent of temperature, the other, k, was easily determined from $(1/m)\ln k$. Therefore, the inactivation ratio of enzymes during preservation at a given temperature could be calculated by substituting the estimated value of the parameters into Eq. 1. The atcivation energy for inactivation of the enzymes was calculated from the slope.

The values of the parameters, activation energy, and the predicted inactivation ratio of the enzymes employed are summarized in Table I. On comparing the predicted inactivation ratios after storage for 1,2 and 3 years, *Rhizopus* lipase, pancreatic α-amylase and diastase appear to be more stable than the others.

TABLE I. Parameters, Activation Energies and Predicted Inactivation Ratios of of Microbial and Pancreatic Lipases and α-Amylases at 25 °C

Enzyme	Water %	Parameter		Activation energy (kcal/mol)	Predicted inactivation ratio		
		m	$(1/m)\ln k$		1 year	2 year	3 year
Lipase							
Aspergillus	7.8	0.91	−7.70	28.6	17.6	30.5	40.9
Rhizopus	8.5	4.33	−8.18	34.1	0.0	0.1	0.6
Candida	7.3	1.43	−6.50	22.9	34.6	68.1	—
Pancreatic	7.9	0.55	−10.07	51.8	11.6	16.5	20.2
Amylase							
Aspergillus	8.6	1.51	−7.47	34.4	9.1	23.4	38.8
Rhizopus	7.8	1.06	−10.08	58.0	1.2	2.5	3.8
Diastase	7.8	2.18	−8.81	50.1	0.2	0.8	1.9

Table II shows the reproducibility of the determined parameter, $(1/m)\ln k$, with pancreatic lipase and *Aspergillus* α-amylase as models. The parameter variation was satisfactorily small.

The inactivation ratios of enzymes in the solid state during storage at 25 and 30°C were determined and compared with the predicted values. The results are shown in Figs. 2—4. It is apparent that the observed results were very close to the predicted values. Therefore,

TABLE II. Reproducibility of the Value of the Parameter $(1/m)\ \ln k$ at 25 °C

Enzyme	Water content %	Number of experiments	$(1/m)\ \ln k$
Pancreatic lipase	7.9	1	−10.07
		2	−10.22
		3	−9.86
		4	−10.05
Asperillus α-amylase	8.6	1	−7.50
		2	−7.45
		3	−7.90
		4	−7.11

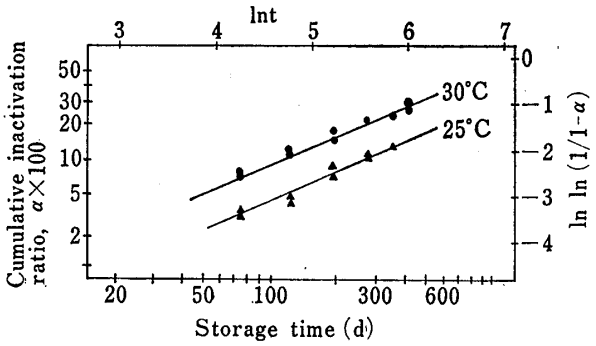

Fig. 2. Comparison of the Experimental Data on Inactivation of *Aspergillus* Lipase in the Solid State (water content, 7.8%) with the Predicted Curves at 25 °C and 30 °C (solid lines)

The experimental data are as follows: (●) at 30°C, (▲) at 25°C.

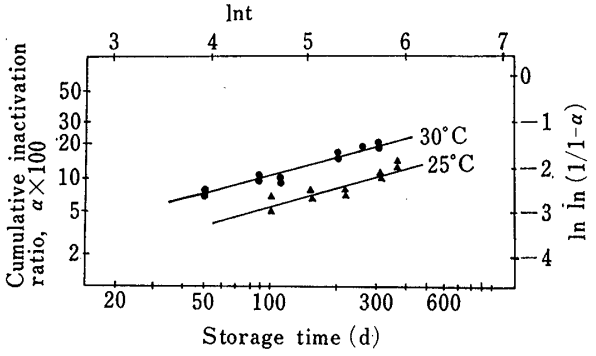

Fig. 3. Comparison of the Experimental Data on Inactivation of Pancreatic Lipase in the Solid State (water content, 7.9%) with the Predicted Curves at 25 °C and 30 °C (solid lines)

The experimental data are as folows: (●) at 30°C, (▲) at 25°C.

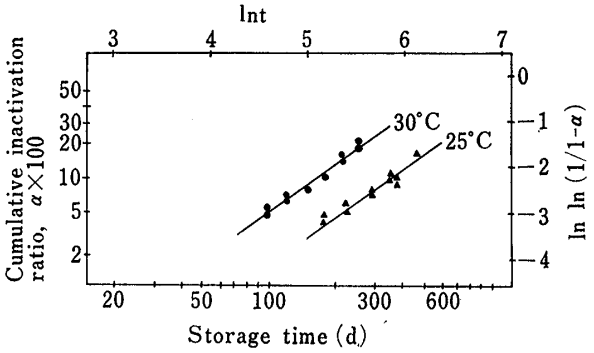

Fig. 4. Comparison of the Experimental Data on Inactivation of *Aspergillus* α-Amylase in the Solid State (water content, 8.6%) with the Predicted Curves at 25 °C and 30 °C (solid lines)

The experimental data are as follows: (●) at 30°C, (▲) at 25°C.

it is possible to assume that our present method for predicting enzyme stability in the solid state is both accurate and useful.

Discussion

An approach for quantitatively predicting the stability of enzymes in the solid state is to use the Weibull distribution function (Eq. 1) and the Arrhenius equation (Eq. 2). Many mathematical theories have been presented,[11–14] but almost all of them, for example zero-, first-, second- and third-order rate equations, the Jander equation,[11] the Kawakita equation,[12] the Prout-Tompkins equation[13] and the Avrami requation,[14] could not fit the inactivation time courses of these experimental results because solid state enzyme inactivation is complicated and affected by many factors. As was reported in the previous paper,[6] the plots of the accumulated inactivation rate of solid state enzymes *versus* time on the Weibull probability paper (within a narrow range) gave a straight regression line. Okusa[7] reported that the Weibull distribution function corresponded to usual reaction rate-equations and could be characterized by two parameters. Of the two parameters m and k, the magnitude of $k^{1/m}$ is proportional to the reaction rate constant. The excellent reproducibility of the two para-

meters and the absence of a temperature effect (<55—60°C) were reported in the previous paper.[6]

The value of the parameters used in the Arrhenius plots was estimated by accelerated testing and graphic calculation. The apparent activation energies, and the estimated values of the parameter $(1/m)\ln k$ at 25°C and 30°C are summarized in Table I and the reproducibility of the parameter is summarized in Table II. The parameter variation was satisfactorily small. In general, it appears from an examination of stability studies in the literature that the selection of temperature levels for accelerated studies has been somewhat arbitrary.

The inactivation ratios of three digestive enzymes in the solid state on storage at 25°C and 30°C as models were calculated by using $(1/m)\ln k$, and represented as a predicted inactivation line in Figs. 2—4. On the other hand, actual values of the inactivation ratios were measured and were in good agreement with the predictions, as shown in Figs. 2—4. The predicted and observed results were treated statistically. The correlation coefficient (r) bebetween the two was 0.954 ($n=60$).

Thus, it is concluded that the stability of enzymes in the solid state may be predicted by the use of mathematically meaningful parameters derived from the Weibull distribution function. An application of our present method to studies on the estimation of the effective life of solid state enzyme preparations should be fruitful.

References and Notes

1) This paper is part CLXXVII of a series entitled "Studies on Enzymes" by M. Sugiura.
2) R. Tardif, *J. Pharm. Sci.*, **54**, 281 (1965).
3) J.T. Carstensen, E.S. Aron, D.C. Spera, and J.J. Vance, *J. Pharm. Sci.*, **55**, 561 (1966).
4) N. Okusa and K. Kinuno, *Yakuzaigaku* (Japanese), **28**, 23 (1968).
5) J.D. Haynes, *J. Pharm. Sci.*, **60**, 927 (1971).
6) M. Sugiura, M. Kurobe, S. Tamura, and S. Ikeda, *J. Pharm. Sci.*, **68**, 1381 (1979).
7) N. Okusa, *Chem. Pharm. Bull.*, **23**, 794 (1975).
8) H.N. Murty, D.L. Biederman, and E.A. Heitz, *J. Phys. Chem.*, **72**, 746 (1968).
9) T. Ogiso and M. Sugiura, *Chem. Pharm. Bull.*, **17**, 1025 (1969).
10) M. Sugiura, *Yakuzaigaku* (Japanese), **28**, 48 (1968).
11) W. Jander, *Z. Inorg. Chem.*, **163**, 1 (1927).
12) K. Kawakita, *Rev. Phys. Chem. Japan*, **14**, 79 (1940).
13) E.G. Prout and F.C. Tompkins, *Trans. Faraday Soc.*, **43**, 148 (1947).
14) M. Avrami, *J. Chem. Phys.*, **7**, 1103 (1939).