Compound Vg reduced the number of *T. canis* larvae in the brains of mice experimentally infected with visceral larva migrans.

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Kinetics of Digestive Enzyme Stability in Solid State I: Application of Weibull Distribution Function to Solid-State Enzyme Inactivation

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Received August 28, 1978, from the *Gifu College of Pharmacy, Mitahora-higashi, Gifu 502, Japan.* Accepted for publication February 9, 1979.

Abstract □ The Weibull distribution function was applied to solid-state enzyme inactivation. On Weibull probability paper (within a narrow range), the plots of the accumulated inactivation ratio of each enzyme *versus* time regressed to a straight line. The parameters m and k, which correspond to the type and rate of the inactivation, were characteristic of each enzyme. The effect of temperature and parameter reproducibility are discussed.

Keyphrases □ Weibull distribution function—application to solid-state enzyme inactivation studies □ Enzyme kinetics—application of Weibull distribution to inactivation studies □ Enzyme inactivation—kinetics, application of Weibull distribution to solid-state studies

Stability is important for drug quality assurance, and reports on drug stability in solid dosage forms have been published (1, 2). The essential requirement in stability studies is the selection of rate equations, which have been determined in practical systems by trial and error.

Some investigations using enzymes in the solid state failed because there was an inherent difficulty in fitting common rate equations to experimental data. Enzyme stability is complicated and is readily affected by factors such as temperature, humidity, and coexisting substances.

This paper[1] describes application of the Weibull distribution function to solid-state enzyme inactivation. The Weibull distribution originally was applied to stability predictions of other solid drugs (3, 4).

THEORETICAL

Efforts have been made to interpret the shelflife of enzymes in solids and solutions and to describe mathematically the inactivation profile with meaningful parameters. Although equations possibly could be derived from empirical treatment of the process, *e.g.*, pseudo-first-order kinetics, no suitable function has been found. Therefore, shelflives rarely have been mentioned in solid-state enzyme inactivation studies.

A general function, applicable to all inactivation curves, was derived by Weibull (5) and discussed in detail (6–8). A concise survey also was reported (9). When applied to enzyme inactivation rate data in the solid state, the Weibull distribution expresses the accumulated inactivation ratio, α, of the enzyme activity at time, t, by:

$$\alpha = 1 - \exp[-(t)^{m/k}] \qquad \text{(Eq. 1)}$$

where the scale parameter, k, defines the time scale of the process and the shape parameter, m, characterizes the curve as an exponential function. The relationship between m and curve shape was discussed previously (8, 9).

Graphical representation of the data according to the Weibull distribution and the practical aspects of linearizing experimental data were reported (6, 7). Equation 1 may be rearranged:

$$\ln \ln (1/1 - \alpha) = \ln k + m \ln t \qquad \text{(Eq. 2)}$$

From Eq. 2, a linear relation is obtained from a ln ln plot of $\ln (1/1 - \alpha)$ *versus* t. The shape parameter, m, is obtained from the slope, and k is obtained from the ordinate value at $t = 1$.

The theoretical correlation between Eq. 2 and chemical kinetics was discussed in detail (4). The equation expressing chemical kinetics varies according to the inactivation mechanism. However, within a narrow range, it is possible to express them by:

$$\frac{d\alpha}{dt} = Kg(a, \alpha, P_i) \qquad \text{(Eq. 3)}$$

where K is the rate constant, a is the initial enzyme activity, and P_i is the parameter independent of time, t. The integrated form of Eq. 3 is:

$$f(a, \alpha, P_i) = Kt \qquad \text{(Eq. 4)}$$

Equations 2 and 4 can be correlated by assuming that logarithms of Eq. 4 are expressed approximately as a linear function of Eq. 2:

$$\ln f(a, \alpha, P_i) = \ln K + \ln t \fallingdotseq A_0 + A_1 \ln \ln (1/1 - \alpha) \qquad \text{(Eq. 5)}$$

$$\ln \ln (1/1 - \alpha) \fallingdotseq 1/A_1 (\ln K - A_0) + 1/A_1 \ln t \qquad \text{(Eq. 6)}$$

[1] This paper is Part CLVII of "Studies on Enzymes" by M. Sugiura.

0022-3549/79/1100-1381$01.00/0

Table I—Regression Line Parameters on Weibull Probability Paper for Lipase and Amylase Inactivation at Various Temperatures

Enzyme	Water, %	Parameter	Temperature 60°	55°	50°	45°	40°
Lipase							
Aspergillus	7.8	m	—	0.82	0.90	0.91	0.93
		$\ln k$	—	−2.74	−3.53	−4.28	−5.13
Rhizopus	8.5	m	4.55	4.73	4.20	4.52	—
		$\ln k$	−10.74	−13.28	−15.56	−20.15	—
Candida	7.3	m	—	2.12	1.40	1.43	1.44
		$\ln k$	—	−5.60	−4.97	−6.00	−6.60
Pancreatic	7.9	m	—	0.68	0.50	0.52	0.52
		$\ln k$	—	−1.69	−2.12	−2.78	−3.47
Amylase							
Aspergillus	8.6	m	—	1.45	1.49	1.57	1.55
		$\ln k$	—	−3.90	−5.20	−6.67	−7.67
Diastase	7.8	m	—	1.69	2.18	2.17	2.21
		$\ln k$	—	−4.90	−7.96	−8.93	−10.68
Pancreatic	7.6	m	—	0.90	0.79	0.83	1.30
		$\ln k$	—	−0.96	−2.50	−4.21	−8.70

Table II—Reproducibility of the Parameters k and m

Enzyme	Water, %	Temperature	Number of Experiments	Parameter m	$\ln k$	$(1/m) \times \ln k$
Aspergillus lipase	4.9	50°	1	0.79	−3.90	−4.91
			2	0.85	−4.09	−4.81
			3	0.68	−3.53	−5.10
			4	0.85	−4.10	−4.82
Aspergillus lipase	11.2	40°	1	1.03	−4.52	−4.39
			2	1.05	−4.60	−4.38
			3	1.08	−4.48	−4.15
Pancreatic lipase	10.1	50°	1	0.50	−2.25	−4.50
			2	0.50	−2.05	−4.10
			3	0.50	−2.12	−4.24

From Eqs. 2 and 6, the relationship between the constants determined on Weibull probability paper and the relationship determined by chemical kinetics are:

$$\ln k = 1/A_1 (\ln K - A_0) \quad \text{(Eq. 7a)}$$

$$m = 1/A_1 \quad \text{(Eq. 7b)}$$

Therefore, from Eq. 7b, m corresponds to the shape of $f(a, \alpha, P_i)$, *i.e.*, the reaction mechanism, and $k^{1/m}$ is proportional to the reaction rate constant. Therefore, the selection of rate equations becomes unnecessary.

EXPERIMENTAL

Enzymes—The enzymes were microbial lipases from *Aspergillus genus*[2], *Rhizopus* sp. NR 400[3], and *Candida cylindracea* nov. sp.[4]; microbial α-amylase from *Aspergillus oryzae*[5]; pancreatin from porcine pancreas[6]; and diastase from malt[7].

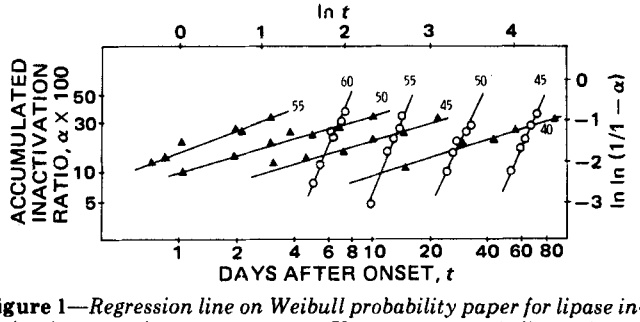

Figure 1—*Regression line on Weibull probability paper for lipase inactivation at various temperatures. Key:* ▲, *pancreatic lipase (water content 7.9%); and* O, Rhizopus *lipase (water content 8.5%).*

[2] Lipase AP, Amano Pharmaceutical Co., Nagoya, Japan.
[3] Lipase Saiken, Osaka Saiken Co., Osaka, Japan.
[4] Lipase MY, Meito Sangyo Co., Nagoya, Japan.
[5] Biodiastase 1000, Amano Pharmaceutical Co., Nagoya, Japan.
[6] Pancreatin, Amano Pharmaceutical Co., Nagoya, Japan.
[7] Diastase, Toyo Jozo Co., Shizuoka, Japan.

Stability Test—All enzymes were tested initially by placing the accurately weighed lyophilizates in open ampuls in a humidity chamber for 1 week. At the end of the equilibrium period, the samples were weighed, and the weight gain was noted. Moisture pickup was expressed as the percent weight gain. The humidity chambers, which were glass desiccators, were kept at ~25°. Constant humidities were obtained by placing appropriate saturated salt solutions in the bottoms of the desiccators.

After the ampuls were sealed, each enzyme was placed in thermostated water baths at 40, 45, 50, 55, and 60°. Samples were removed at appropriate periods, and the remaining activity was determined.

Enzyme Activity Assay—The activities of lipases and α-amylases were determined according to a reported procedure (10, 11). The remaining activity was expressed as a ratio relative to that of the enzyme stored in a refrigerator.

RESULTS AND DISCUSSION

The inactivation behavior of pancreatic lipase and *Rhizopus* lipase at various temperatures is shown in Fig. 1; the accumulated inactivation ratios were plotted *versus* time according to the linearized form of Eq. 2. Within a narrow range (inactivation ratio 5–30%), the inactivation data plots yielded a straight regression line. The Weibull distribution function distorted the original scale of the observation greatly; in particular, deviations occurring in the lower and the upper tail were overemphasized compared to those in the middle of the plot.

The same stress test was carried out with lipase and α-amylase samples. The inactivation ratio plot of each enzyme also regressed to a straight line, which confirmed that enzyme inactivation could be followed by the Weibull distribution function. The parameters m and k were determined by a graphic calculation (Table I). Table I shows the specific parameters that characterize the inactivation profile for each enzyme. Temperatures of <55–60° had no influence on the parameters, which indicated that the stress test could be applied to solid enzyme inactivation studies during preservation.

The finding that most of the m values for each enzyme differed from those of common rate equations, estimated at ~1.0 (4), confirmed an inherent difficulty in fitting other common rate equations to experimental data and thereby interpreting enzyme stabilities.

Table II shows the reproducibility of the parameters with pancreatic lipase and *Rhizopus* lipase as a model. Table II shows that the resultant parameter variation was satisfactorily small. Therefore, the Weibull distribution is applicable to solid-state enzyme inactivation studies in practical fields.

Enzyme inactivation in aqueous systems has been studied extensively with regard to protein structures and biochemical functions (12–14). The present study was confined to application of the Weibull distribution function to solid-state enzyme inactivation. Enzyme inactivation in the solid state could be described by a straight regression line in terms of meaningful parameters. The excellent parameter reproducibility and the absence of temperature influence ensure that this function will be useful in further work involving the quantitative prediction of solid-state enzyme stabilities.

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Evaluation of 1-(3,4-Dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one Hydrochloride Effect on Nucleic Acid and Protein Syntheses Using Murine Leukemia L-1210 Cells

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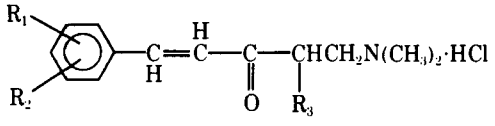
Received March 2, 1979, from the **Department of Physiological Sciences, Western College of Veterinary Medicine, and the* ‡*College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, S7N 0W0.* Accepted for publication May 11, 1979.

Abstract □ Several Mannich bases derived from conjugated styryl ketones were shown to have potent cytotoxicity toward murine leukemia L-1210 cells and Walker 256 carcinosarcoma cells in culture. The most cytotoxic derivative, (*E*)-1-(3,4-dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one hydrochloride, profoundly inhibited the incorporation of tritiated leucine into protein(s) and tritiated deoxythymidine into DNA at concentrations of 0.79–1.32 μM in L-1210 cells. At higher concentrations, incorporation of tritiated uridine into RNA and tritiated deoxyuridine into DNA was inhibited to a lesser degree. This compound failed to inhibit the enzymes thymidylate synthetase or dihydrofolate reductase up to a concentration of 10^{-4} M and was ineffective in retarding the growth of the Walker 256 carcinosarcoma in rats.

Keyphrases □ Mannich bases—derived from conjugated styryl ketones, cytotoxicity, murine leukemia L-1210 and Walker 256 carcinosarcoma cells, *in vitro* and *in vivo* □ Antineoplastic agents, potential—Mannich bases derived from conjugated styryl ketones, cytotoxicity, murine leukemia L-1210 and Walker 256 carcinosarcoma cells, *in vitro* and *in vivo* □ 1-(3,4-Dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one hydrochloride—cytotoxicity against murine leukemia L-1210 and Walker 256 carcinosarcoma cells, *in vitro* and *in vivo*

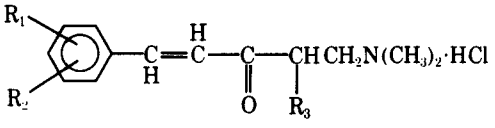
Several Mannich bases derived from acyclic α,β-unsaturated ketones (I) have shown antitumor and cytotoxic activities (1–3). Previous studies showed a correlation between cytotoxicity and murine toxicity with mitochondrial function disturbance (4). With the representative Mannich bases II*b*–II*e*, competition with coenzyme Q_{10} occurred (5). The purpose of this study was to discover whether these Mannich bases act at sites other than mitochondria or whether the biological activity observed is due solely to the effect on mitochondria.

Since series I compounds may be regarded as biological alkylating agents, a class of compounds known to interfere with the syntheses of DNA and RNA (6–8) as well as protein (9, 10), the effect on the syntheses of these biological macromolecules is reported.



I

$R_1 = R_2 = H, Cl$
$R_3 = H, CH_3, (CH_2)_4CH_3$



II

II*a*: $R_1 = R_2 = R_3 = H$
II*b*: $R_1 = R_2 = H, R_3 = (CH_2)_4CH_3$
II*c*: $R_1 = 2\text{-Cl}, R_2 = 4\text{-Cl}, R_3 = (CH_2)_4CH_3$
II*d*: $R_1 = 2\text{-Cl}, R_2 = 6\text{-Cl}, R_3 = (CH_2)_4CH_3$
II*e*: $R_1 = 3\text{-Cl}, R_2 = 4\text{-Cl}, R_3 = (CH_2)_4CH_3$

RESULTS AND DISCUSSION

Earlier work from these laboratories, which showed that the established biological alkylating agents chlorambucil and cyclophosphamide inhibited DNA synthesis, employed murine leukemia L-1210 and Walker ascites carcinoma 256 cells (11, 12). The first consideration in the present study was to establish whether representative compounds in Series I were cytotoxic to these two cell lines. Since II*a*–II*e* had previously demonstrated activity in the KB screen (4, 5), an *in vitro* screen using human epidermoid carcinoma of the nasopharynx in Eagle's medium, these compounds were examined for cytotoxicity against the L-1210 and Walker 256 cells. Both tumors showed essentially the same sensitivity to II*a*–II*e* with ID_{50} values (inhibitory doses for 50% reduction in cell numbers) in the range of 3.44–3.96 μM. The most potent compound against L-1210 cells was II*e*, with an ID_{50} of 3.46 μM (Fig. 1). This compound was used in subsequent studies.

Some reactions involved in the DNA, RNA, and protein syntheses are summarized in Scheme I. During the exponential growth phase, cells preferentially utilize deoxyuridine for the *de novo* synthesis of deoxythymidine monophosphate (13). This reaction is catalyzed by thymidylate synthetase. An alternative route for deoxythymidine monophosphate synthesis is by direct phosphorylation of deoxythymidine. Deoxythymidine monophosphate, after conversion to its corresponding triphosphate, is utilized for DNA synthesis (13). Thus, the effect of compounds on DNA synthesis can be monitored by studying the incorporation of either tritiated deoxythymidine or tritiated deoxyuridine.

Similarly, the effects of biologically active compounds on RNA synthesis can be investigated by studying their effects on the incorporation

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