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Colorimetric Determination in the Frozen State with Dual-Wavelength Spectrophotometer.[1] Application to the Kinetics of Hydrolysis of *p*-Nitrophenyl Acetate

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It is generally practised to store drugs and foods in the dark, cool places. Especially frozen foods are kept in the frozen state to prolong the period of their usability. Though it is commonly acknowledged that the chemical reaction proceeds more slowly at the lower temperature than at the higher one, the thoughts should not be adapted to the frozen state beyond the range of liquid state. It was already known in 1939 that a chemical reaction could also occur in the frozen state.[3] Moreover quite a few cases were found that the chemical reactions proceed more rapidly in the frozen state than in the solution. Grant, *et al.*[4] studied the base-catalyzed decomposition of penicillin and found that penicillin solutions containing imidazole or histidine lost antimicrobial activity when stored at −18°, but not when incubated for 17 hours at 38°. The hydroxylaminolysis of amides[5] and amino acid esters[6] in the frozen state at −18° was faster than in the solution at 0° or 22°. Bruice, *et al.*[7] showed that the hydrolytic rate constants of acetic anhydride, *p*-propiolactone and *p*-nitrophenyl acetate in water at −10° (extrapolated employing values of $\Delta H^{\ddagger}$ determined in water) were smaller than at −10° in ice. They attributed these phenomena to the "concentration effect." In the process of freezing the reactant might be excluded from ice crystals and concentrate in the "liquid region" or "puddle"[8] which co-exist among ice phase. If there are any catalyst, both the reactant and catalyst converge together in the puddle and the reaction proceeds more rapidly in the frozen state than in solution (the values are obtained by extrapolation). The co-existence of salts makes the liquid region wider and therefore the concentration of catalyst becomes thinner which reflects to slower reaction. If there is no catalyst the concentration of substrate does not affect the reaction rate. Concerning the enhancement of reaction rates there are a few possible explanations besides stated above, *e.g.*, compulsory rearrangement[4] of the reactants and catalysts at the freezing process, the exceptionally high proton mobility in ice,[4] the effect of crystal structure of ice and/or crystal imperfections,[5,9] the change of dielectric constant[6] followed by phase transformation, and the existence of catalytically active sites on the ice surface.[6,10]

There is an ambiguity in the definition of "frozen state." As pointed out by Pincock,[8] freezing point is definite in one component system (Fig. 1-a), namely it is the point where liquid phase transforms into solid phase, or *vice versa*. The systems where the chemical reaction kinetics are studied consist of more than two components and belong to the system

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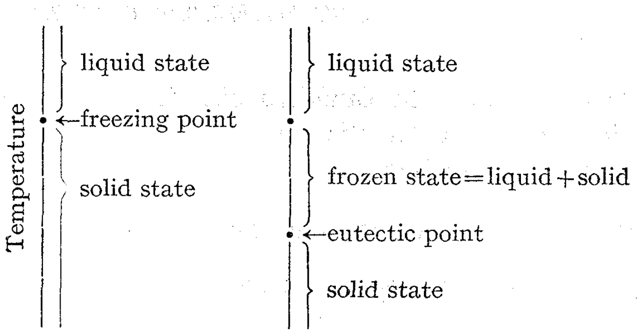

(a) one component (b) two or more components

Fig. 1. Diagram of the Range of Frozen State[8)]

expressed in Fig. 1-b. It implies that a frozen state exists in the range of temperatures below the freezing point and above the eutectic point, *i.e.*, where solid is in equilibrium with a liquid phase. This concept will be used throughout this report.

In the reaction of ethylene chlorohydrine[11)] with NaOH in frozen aqueous solutions, the frozen system at −5° was 99.9% solid and the reaction was accelerated by a factor of 1000. However the important part of the system studied here was the normal but concentrated liquid which made up only *ca.* 0.1% of the total volume. According to Zaromb, *et al.*[12)] one can expect up to 20% apparent incorporation of the solute (NH_4F) into ice crystals, and Bruice, *et al.*[13)] also stated that the solute (morpholine) was included in ice crystals. It is not easy, anyhow, to elucidate the reaction mechanism in the frozen state.

The measurement of reaction rate in the frozen state are usually done by thawing the systems. But thawing is not a desirable procedure and makes it impossible to measure the reaction rate continuously. In this report a method without thawing the system was proposed to determine the reaction rate by colorimetry measuring the increase of product or the decrease of reactant with dual-wavelength spectrophotometer.

In the freezing process the volume of the solution increases or decreases with change of temperature according to its components, therefore the frozen sample to be measured by spectrophotometry with uniformal thickness are seldom obtainable. This makes transmittance and/or dispersion of light unpredictable from sample to sample. These effects by thickness or surface condition of sample were normalized by combinations of dual-wavelength spectrophotometries: one measurement to normalize these effects and the other to determine the concentration of reactant or product for each sample.

This method was applied to measure the hydrolytic reaction rate of *p*-nitrophenyl acetate.

Theoretical

In a system, solute a (molar concentration C_a), solute b (molar concentration C_b), and solvent c, let the apparent absorbances in the frozen state at the wavelength λ_1, λ_2, λ_3, and λ_4 be $E_{\lambda 1}$, $E_{\lambda 2}$, $E_{\lambda 3}$, and $E_{\lambda 4}$, respectively. Then the following equation is obtained,

$$E_{\lambda_i} = C_a \varepsilon_{a\lambda_i} l + C_b \varepsilon_{b\lambda_i} l + A_i$$

where $\varepsilon_{a\lambda i}$ and $\varepsilon_{b\lambda i}$ are molar absorptivities of component a and b at wavelength λ_i, respectively, and A_i is the apparent absorbance of frozen solvent at wavelength λ_i and l is the apparent path length. A_i is more affected by the conditions of sample surface than path length l.

Dual-wavelength spectrophotometries are done at λ_1—λ_2, and λ_3—λ_4, then,

$$E_{\lambda_1} - E_{\lambda_2} = C_a l(\varepsilon_{a\lambda_1} - \varepsilon_{a\lambda_2}) + C_b l(\varepsilon_{b\lambda_1} - \varepsilon_{b\lambda_2}) + (A_1 - A_2)$$
$$E_{\lambda_3} - E_{\lambda_4} = C_a l(\varepsilon_{a\lambda_3} - \varepsilon_{a\lambda_4}) + C_b l(\varepsilon_{b\lambda_3} - \varepsilon_{b\lambda_4}) + (A_3 - A_4)$$

By setting the two wavelengths close, following conditions are met,

$$A_1 - A_2 \fallingdotseq 0, \qquad A_3 - A_4 \fallingdotseq 0$$

Then

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11) R.E. Pincock and T.E. Kinovsky, *J. Am. Chem. Soc.*, **88**, 4455 (1966).
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$$\frac{E_{\lambda_1}-E_{\lambda_2}}{E_{\lambda_3}-E_{\lambda_4}}=\frac{C_a(\varepsilon_{a\lambda_1}-\varepsilon_{a\lambda_2})+C_b(\varepsilon_{b\lambda_1}-\varepsilon_{b\lambda_2})}{C_a(\varepsilon_{a\lambda_3}-\varepsilon_{a\lambda_4})+C_b(\varepsilon_{b\lambda_3}-\varepsilon_{b\lambda_4})}$$

is obtained.

If the following conditions could be met, such as λ_1—λ_2 where component b has little absorbance in the neighborhood of a peak by component a being taken into consideration of concentrations of components a and b, and λ_3—λ_4 where the conditions reversed stated above. These mean

$$C_a(\varepsilon_{a\lambda_1}-\varepsilon_{a\lambda_2}) \gg C_b(\varepsilon_{b\lambda_1}-\varepsilon_{b\lambda_2})$$

$$C_a(\varepsilon_{a\lambda_3}-\varepsilon_{a\lambda_4}) \ll C_b(\varepsilon_{b\lambda_3}-\varepsilon_{b\lambda_4})$$

then the equation reduces to

$$\frac{E_{\lambda_1}-E_{\lambda_2}}{E_{\lambda_3}-E_{\lambda_4}}=\frac{C_a(\varepsilon_{a\lambda_1}-\varepsilon_{a\lambda_2})}{C_b(\varepsilon_{b\lambda_3}-\varepsilon_{b\lambda_4})}=k\frac{C_a}{C_b}$$

This implies that the increase or decrease of component a could be measured by normalizing with index of b cancelling the effects by path length l and/or by sample surface (Fig. 2).

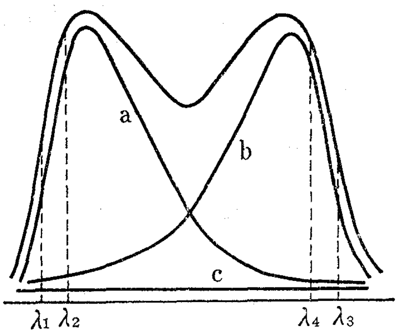

Fig. 2. Schematic Diagram of Colorimetric Determination in Frozen State

$$E_{\lambda_i}=C_a\varepsilon_{a\lambda_i}l+C_b\varepsilon_{b\lambda_i}l+A_i$$

For detail, see text under Theoretical.

Experimental

Reagents——Dimethyl sulfoxide (DMSO), acetonitrile, Orange I (OI), Naphthol Yellow S (NY), Brilliant Blue 6b (BB) were used as received. Trishydroxymethylaminomethane (tris buffer) was recrystallized from 20(v/v)% EtOH–H_2O and desiccated under vacuum. *p*-Nitrophenyl acetate (NPA) was recrystallized from ethylether. All reagents except DMSO from Wako Pure Chem. Ind., Ltd., were purchased from Tokyo Kasei Kogyo Co., Ltd.

Apparatus——Hitachi 356 dual-wavelength spectrophotometer equipped with constant temperature cell holder connected to a thermostated circulator (Lauda K2RD) was used. The temperature of −5° was maintained unless otherwise stated. pH measurements were made with a pH meter 28 (Radiometer).

Method——NY and BB-OI were dissolved in the mixture of 96% DMSO–H_2O (freezing point 8.6°, This solvent was found most manipulatable so far.). Into a photometric cell having the light path length of 1 or 2 mm, 3×10^{-4}M NY solution was introduced and freezed in a constant temperature bath at −5° for 15 min. Dual-wavelength spectrophotometries were carried out at $\lambda_1$470—$\lambda_2$460 nm and $\lambda_3$405—$\lambda_4$395 nm and the ratio Abs(470—460)/Abs(405—395) was obtained.

From the stock solutions of 2×10^{-3}M BB or 4×10^{-3}M OI, solutions were mixed appropriately to produce $2\sim4\times10^{-4}$M as for BB and $4\sim8\times10^{-4}$M as for OI then introduced into a cell having 1, 2, or 3 mm path length and frozen as stated above. Measurements were made at 670—680 nm and 390—400 nm. The ratios Abs(670—680)/Abs(390—400) were obtained.

BB-NPA——NPA was dissolved in acetonitrile as a stock solution. With HCl 0.1M tris buffer solution was adjusted to pH 9.93. As the reaction medium 3×10^{-4}M BB in 0.1M tris buffer solution was used. With this medium 0.1 ml of 0.1M NPA was diluted to 5 ml and introduced into a precooled glass cell which was handmade with thickness of 2.3 mm and freezed in a freezer at −23° for 30 min. Then the cell was transferred into a thermostat and kept there at −5° and dual-wavelength spectrophotometries were carried out at given intervals at 420—430 nm and 670—680 nm. Thus the hydrolytic reaction rate was calculated from the increase of absorbance due to the product (*p*-nitrophenol) from the value measured at 420—430 nm and normalized by the value at 670—680 nm.

The rate of the hydrolysis of NPA in solutions were measured with absorbance increase at 400 nm at appropriate intervals.

Result and Discussion

The absorbances of frozen solvent 96(v/v)%DMSO–H_2O measured by dual-wavelength spectrophotometry were shown in Table I.

The apparent absorbances were not depended on the apparent thickness of light path as seen in the Table I but seemed to be affected by the surface condition of the sample through light scattering. Anyhow Abs (670—680) and Abs (390—400) were found, as anticipated, small and negligible in the calculation of the absorbance ratio.

NY was used because it has two peaks in its absorption spectrum not separated too much and measured both ways in solution and in frozen state. The results were compiled in Table II

Table I. Absorbance of 96 (v/v) % Dimethyl Sulfoxide in Frozen State Measured by Dual-Wavelength Spectrophotometry

Path length[a]	Abs. (670—680)[b]	Abs. (390—400)	Path length[a]	Abs. (670—680)	Abs. (390—400)	Path length[a]	Abs. (670—680)	Abs. (390—400)
3 mm	0.012	0.015	2 mm	0.026	0.028	1 mm	0.010	0.020
	0.026	0.028		0.036	0.030		0.002	0.000
	0.015	0.014		0.012	0.016		0.015	0.024
	0.001	0.002		0.030	0.033		0.010	0.012
	0.000	0.002		0.027	0.030		0.002	0.004
	0.010	0.013		0.005	0.001		0.016	0.017
	0.015	0.011		0.050	0.047		0.038	0.027
	0.010	0.013		0.000	0.000		0.016	0.015
	0.035	0.035		0.011	0.009		0.016	0.017
	0.024	0.027		0.005	0.009		0.001	0.002

a) apparent b) values in the parentheses imply nm

Table II. Absorbances and Absorbance Ratios of Naphthol Yellow S in Liquid and Frozen State (3×10^{-4}M)

Path length	Abs. (470—460)[a]	Abs. (405—395)	$\frac{\text{Abs. (470—460)}}{\text{Abs. (405—395)}}$	
		Liquid		
2 mm	0.395	0.315	1.25	$\bar{x}$ 1.321
	0.417	0.326	1.28	V 0.006
	0.396	0.286	1.38	$\sqrt{V}$ 0.076
	0.376	0.300	1.25	S.E. 0.029
	0.376	0.264	1.42	
	0.378	0.298	1.27	
	0.369	0.264	1.40	
1 mm	0.208	0.150	1.39	$\bar{x}$ 1.346
	0.218	0.157	1.39	V 0.005
	0.201	0.162	1.24	$\sqrt{V}$ 0.070
	0.195	0.139	1.40	S.E. 0.026
	0.190	0.150	1.27	
	0.200	0.142	1.41	
	0.190	0.144	1.32	
		Frozen state[b]		
2 mm	0.478	0.349	1.37	$\bar{x}$ 1.497
	0.536	0.362	1.48	V 0.004
	0.507	0.340	1.49	$\sqrt{V}$ 0.064
	0.458	0.300	1.53	S.E. 0.024
	0.420	0.268	1.57	
	0.470	0.310	1.52	
	0.443	0.291	1.52	
1 mm	0.265	0.170	1.56	$\bar{x}$ 1.493
	0.277	0.201	1.38	V 0.020
	0.268	0.179	1.50	$\sqrt{V}$ 0.141
	0.237	0.160	1.48	S.E. 0.053
	0.241	0.161	1.50	
	0.271	0.210	1.29	
	0.228	0.131	1.74	

a) values in the parentheses imply nm b) apparent path length
$\bar{x}$: mean V: variance S.E.: standard error

and showed that Abs (470—460)/Abs (405—395) were constant independent of conditions namely in solution or in frozen state and of light path length.

As there was only one species of the chromophore in the system the ratio was constant independent of NY concentration. The spectra of NY in solution and in the frozen state were shown in Fig. 3 and were almost superimposable.

Then two dyes were employed simultaneously. They were BB and OI and their peaks in the absorption spectra were separated enough and their spectra were almost the same in solution and in frozen state and there was no detectable interaction between these dyes.

In solution the value of Abs (670—680)/Abs (390—400) were constant indifferent of light path length. According to Taborsky[14] solutes might disperse unevenly in a cell in the process of freezing. This means there is a possibility that the concentrations of dyes in the light path might be different from sample to sample. Though the variation in the values within the frozen state was greater than in solution but insignificant upon *t*-test of mean values with 5% risk. These results were compiled in Table III. The values of absorbance ratio were constant indifferent its light path length if they were in the same phase. When the concentration of OI was in the range of 4—6×10^{-4}M the ratio was independent of conditions but when the concentration of OI was 8×10^{-4}M the values of ratio might not be constant.

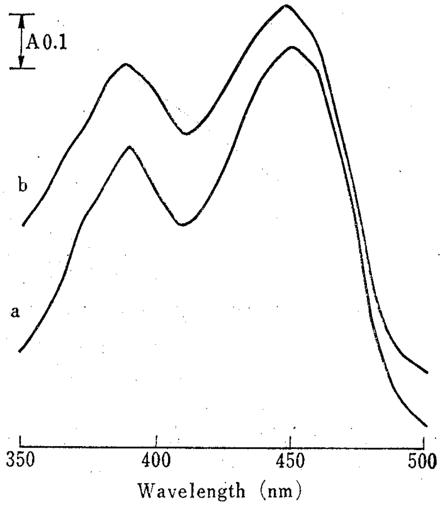

Fig. 3. Absorption Spectra of Naphthol Yellow S in 96 (v/v) % Dimethyl Sulfoxide

a: liquid b: frozen state

Table III. The Means of Ratio of Abs (670—680) to Abs (390—400) in Liquid and Frozen State

BB		OI					
		4×10^{-4}M		6×10^{-4}M		8×10^{-4}M	
		Liquid	Frozen[a]	Liquid	Frozen[a]	Liquid	Frozen[a]
2×10^{-4}M	1 mm			1.24	1.19	0.93	1.13
	2 mm	1.80	1.87	1.20	1.19	1.00	1.24
	3 mm	1.86	1.85				
3×10^{-4}M	1 mm			1.83	1.92	1.44	1.66
	2 mm	2.64	2.66	1.86	1.92	1.49	1.79
	3 mm	2.62	2.68				
4×10^{-4}M	1 mm			2.39	2.31	1.78	1.97
	2 mm			2.35	2.27	1.83	2.02

BB: Brilliant Blue 6b OI: Orange I

a) apparent path length

Though within some limitations that it is necessary to choose a proper substance to normalization, the concentration ratio of normalizing substance to the reactant, or the combination of concentration and light path length, and so on, this method was shown to be applicable to the reaction kinetics in frozen state.

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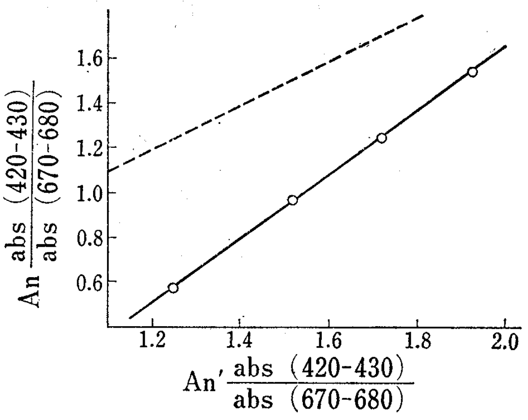

Fig. 4. A Plot of Data Obtained from Hydrolysis of *p*-Nitrophenyl Acetate-Brilliant Blue 6b at −5° in Frozen State According to
$A_n = A_\infty[1-\exp(k\Delta t)] + A_n' \exp(k\Delta t)$
The dotted line is the bisectrix.

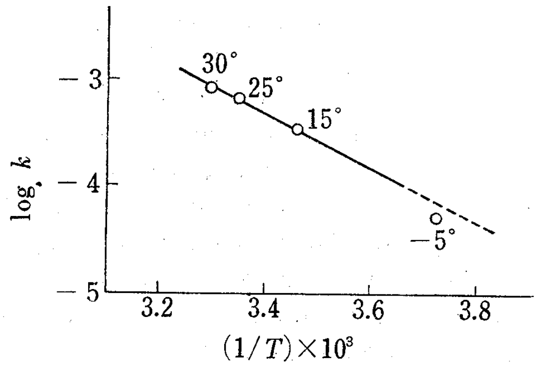

Fig. 5. Arrhenius Plot of Hydrolysis of *p*-Nitrophenyl Acetate-Brilliant Blue 6b

The hydrolytic reaction rate of NPA in frozen state was measured by this method. BB was utilized as a normalizing substance. A_n and A_n' were plotted according to the equation[15–18]

$$A_n = A_\infty[1-\exp(k\Delta t)] + A_n' \exp(k\Delta t)$$

where A_n: reading of absorbance (normalized) at time t, k: rate constant, and Δt: time increment. Δt was set at 120 min here (Fig. 4).

Rate constant k was determined as 3.20×10^{-3} min^{-1} from the relation of (slope) $= \exp(k\Delta t)$.

Then the Arrhenius plot was made with this value and the values from in solutions. The dotted line in Fig. 5 shows the extrapolated value from liquid phase. The value obtained in this report was about a half of extrapolated one.

Pincock[11,19] and Bruice[7] reported that the existence of third substance in the system could suppress the reaction. In this study index substance BB and/or tris buffer could be the ones which hindered the hydrolysis of NPA. Or difference of phase might cause the different mechanism of the reaction. Further investigations are needed to draw some conclusion about this point.

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