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Dehydrogenation of Ethylalcohol Catalyzed by Alcoholdehydrogenase Under High Pressure

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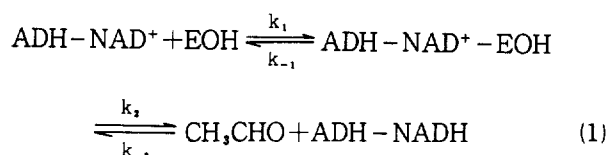
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A pressure effect of the dehydrogenation of ethylalcohol catalyzed by alcoholdehydrogenase was observed in Tris-HCl buffer, pH 8.8 from 25 °C to 35 °C under high pressure system by using our new theory. The theory makes it possible for us to obtain all rate and equilibrium constants for each step of all enzymatic reaction with a single intermediate. We had enthalpy and volume profiles of the dehydrogenation to suggest a detail and reasonable mechanism of the reaction. In these profiles, both enthalpy and entropy of the reaction are positive and their values decrease with enhancing pressure. It means that the first step is endothermic reaction, and its strength decrease with elevating pressure. At the same time, all activation entropies have large negative values, which prove that not only a ternary complex has a more ordered structure at transition state, but also water molecules make a iceberg close by the activated complex. In addition to this fact, the first and second step equilibrium states are controlled by enthalpy. The first step kinetic state is controlled by enthalpy but the second step kinetic state is controlled by entropy.

Introduction

The steady-state approximation provides a many convenient solutions to the kinetic equations for studing enzyme kinetics. But inherent disadvantage of the approximation is that little information about a transition of enzymatic reaction is obtained[1-10]. To remove this weakness we have suggested a new method which were able to give all rate constants for each step of all enzymatic reactions with a single intermediate[11]. The new method was applied to the dehydrogenation of ethylalcohol catalyzed by yeast alcoholdehydrogenase as follows;

$$ADH-NAD^+ + EOH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ADH-NAD^+ - EOH$$

$$\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CH_3CHO + ADH - NADH \qquad (1)$$

where NAD^+, $NADH$ and EOH are β-nicotinamide adenine dinucleotide, its reduced form and ethylalcohol respectively.

In this paper we will study the pressure effect of Eq(1). If the total substrate concentration, [EOH], is much greater than the total concentration of holoenzyme which is a complex between ADH and NAD^+, the value of [EOH] can be assumed to remain constant during the course of the reaction. The reaction now becomes pseudo first order and rate equation can be written. as

$$v = d[NADH]/dt = k[EOH][ADH-NAD^+]$$
$$= k_o[ADH-NAD^+] \qquad (2)$$

where k_o stands for an observed pseudo first order rate constant. The k_o values can be obtained from the following equation[12-13].

$$\ln(A_\infty - A_t) = -k_o t + \ln(A_\infty - A_o) \qquad (3)$$

where A's represent the UV absorbances of NADH and their

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subscripts do the reaction times respectively.

The conventional Lineweaver-Burk plot[14] which is the most common plot in the steady-state approximation is given by

$$1/v = 1/v_m + (K_M/v_m)/[EOH] \qquad (4)$$

where K_M and v_m are Michaelis-Menten constant defined by $(k_{-1} + k_2)/k_1$ and maximum velocity respectively.

A new type of Lineweaver-Burk[15] was derived in detail as follows;

$$1/k_o = 1/k_m + (K_M/k_m)/[EOH] \qquad (5)$$

where k_m is called to the maximum rate constant[15] defined by

$$k_m = v_m/[ADH-NAD^+] \qquad (6)$$

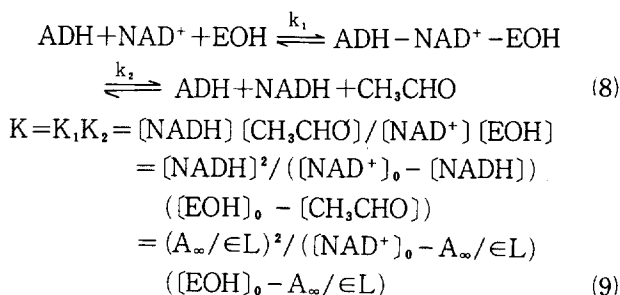where v_m stand for the maximum velocity of the conventional Lineweaver-Burk plot.

An another new important equation was derived in detail by using the presteady state approximation in our previous paper[15].

$$(A_\infty - A_t)/\in L = - (v_m k_2/k_m)t + (k_m k_2/v_m k_1)/[EOH]_o \qquad (7)$$

where $\in$ and L represent the molar extinction coefficient of NADH and the transmitting thickness of UV curvette respectively.

From a slope and intercept of Eq(7), we can calculate k_1 and k_2 respectively having known the values of v_m and k_m in Eq(4) and (5) respectively.

The overall equilibrium constant, K of Eq(8) can be obtain from Eq(9).

$$ADH+NAD^+ +EOH \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ADH-NAD^+ -EOH$$

$$\rightleftharpoons ADH+NADH+CH_3CHO \qquad (8)$$

$$\begin{aligned} K = K_1 K_2 &= [NADH][CH_3CHO]/[NAD^+][EOH] \\ &= [NADH]^2/([NAD^+]_0 - [NADH]) \\ &\quad ([EOH]_0 - [CH_3CHO]) \\ &= (A_\infty/\in L)^2/([NAD^+]_0 - A_\infty/\in L) \\ &\quad ([EOH]_0 - A_\infty/\in L) \qquad (9) \end{aligned}$$

Accordingly, from Eq(4), (5), (7), (8) and K_M, we could have all kinetic and equilibrium constants of all enzymatic reactions with a single intermediate mechanism under the condition that Eq(1) is good approximate to Eq(8).

All kinetic and thermodynamic parameters corresponding to these all constants can be calculated from well-known equations like Eq(10) and (11) respectively.

$$\begin{aligned} \Delta H^0 &= (RT_1 T_2/(T_2-T_1))\ln (K_{T_2}/K_{T_1}) \\ \Delta S^0 &= (\Delta H^0 - \Delta G^0)/T \\ \Delta V^0 &= -RT(\partial \ln K/\partial P)_T \qquad (10) \end{aligned}$$

where ΔH^0, ΔS^0, ΔG^0, ΔV^0 are enthalpy, entropy, Gibbs free energy and volume change of Eq(8) at equilibrium state respectively, and K_{T_1} and K_{T_2} represent equilibrium constants at temperature, T_1 and T_2 respectively. ΔV^0 has the differential equation instead of intergral equation because experiment was not run at two temperatures and constant pressure as ΔH^0, ΔS^0 and ΔG^0. The activation parameters cor-

responding to Eq(10) are represented as Eq(11).

$$\begin{aligned} \Delta H^* &= (RT_1 T_2/(T_2-T_1))\ln (K_{T_2}/K_{T_1}) \\ \Delta S^* &= (\Delta H^* - \Delta G^*)/T \\ \Delta V^* &= -RT(\partial \ln k/\partial P)_T \qquad (11) \end{aligned}$$

where ΔH^*, ΔS^*, ΔG^* and ΔV^* stand for activation enthalpy, free energy and volume of Eq(1) at transition state respectively, and K_{T_1}, K_{T_2}, k, k' and h mean rate constants at temperature, T_1, T_2 arbitrary temperature, Boltzman constant and Plank constant respectively.

To determine whether the reaction of Eq(8) is controlled by enthalpy and entropy, we must calculate isoequilibrium and isokinetic temperature of the reaction from Eq(12) and (13)[17].

$$\delta \Delta H = ((T\alpha - 1)/\alpha)\delta \Delta S = \bar{\beta}\delta \Delta S \qquad (12)$$

where $\delta \Delta H$ and $\delta \Delta S$ represent their change between 1 and P bars respectively. The isoequilibrium temperature, $\bar{\beta}$ is the function of isobaric volume expansivity, α and the isothermal compressibility, .

$$\delta \Delta H^* = ((T\alpha^* - 1)/\alpha^*)\delta \Delta S^* = \bar{\beta}\delta \Delta S^* \qquad (13)$$

where a subscript, double dagger means a activation state and other notations are the same as Eq(12). The isokinetic temperature, β^* is also the function of the activation isobaric volume expansivity, α^* and the activation isothermal compressibility, β^*.

Experimental Procedures

Materials. Yeast Alcoholdehydrogenase, ADH and its cofactors, β-nicotinamide adenine dinucleotide, β-NAD$^+$(its abbreviation NAD$^+$) and β-nicotinamide adenine dinucleotide reduced form, β-NADH(its abbreviation NADH) were purchased from the Sigma Chemical Company, U.S.A. as crystalline powder, Absolute ethylalcohol was obtained from Merck, West Germany. The enzyme and its cofactors were dessicated below 0 °C before reconstituting. For reconstituting 1 mg of the enzyme was dissolved in 1 ml of ice cold water of which temperature was below 0 °C and stored with frozen to maintain its activity as long as possible[18,19].

High Pressure Cell. A new type of Lentz Cell was made. It was bran ched with stainless steel tube fitted with stopper. The branched tube is used when experimenter adds the ADH solution into the cell by using microsyringe to initiate kinetic run. The hand operated pressure pump was described in the previous paper[20]. A liquid separating vessel was made to separate sample solution from a pressure transmitting fluid. The vessel was connected between the cell and pressure pump with high pressure steel tube.

Kinetic Measurement. Stock solutions of separate were freshly prepared by dissolving ethanol in 0.1 M Tris-HCl buffer pH 8.8 having contained NAD$^+$. Kinetic runs were initiated by addition of 10 μl ADH solution in the high pressure cell filled with 1.5 ml stock solution by microsyringe. At that very moment, this kinetic system was pressed to a constant pressure by a rapid opening the pressure preventing valve. Accordingly an assay solution and 10 μl ADH solution. A typical experimental data were written in Table 1. The data and a residual data which were not shown in Table 1 were used to calculate various rate constants and equilibrium constants Eq(8).

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Table 1. A Typical Experimental Data of the Dehydrogenation of Ethylalcohol by ADH-NAD$^+$ [a]

t(sec.)	A_t	A_∞-A_t	ln(A_∞-A_t)	((A_∞-A_t)/εL) × 10^{5}M
60	0.209	0.492	-0.709	7.91
120	0.255	0.466	-0.764	7.49
180	0.295	0.406	-0.901	6.53
240	0.327	0.375	-0.981	6.03
300	0.356	0.345	-1.06	5.55
360	0.387	0.314	-1.16	5.05
420	0.411	0.290	-1.24	4.66
480	0.435	0.266	-1.32	4.28
∞	0.701			

[a] For details, see Experimental Procedures. The typical experimental data come when the substrate concentration is 2.5 × 10^{-3}M at 25 °C and 900 bars. The molar extinction coefficient, ε of NADH at 340nm was 6220 dm^{3}mol^{-1}. The data agree with the linear equation such as Eq(3) and Eq(7) as shown in Figure 3.

Result and Discussion

It was manifested experimentally that the initiation step of the reaction of Eq(1) had to be the step in which holoenzyme appears. Therefore, it is also interesting for us to evaluate the binding constant between ADH and NAD$^+$. It is reasonable that we can assume the first step of Eq(8) contains the binding step and so the first step equilibrium constant, K_1 is a binding constant. A behaviour of K_1 is going to be explained later.

The observed rate, v and rate constant k_o were calculated from Eq(2) and (3) by using the data listed in Table 1 to be shown in Table 2. The values of v and k_o increase with elevating pressure and temperature. This fact proves that there is no deactivation of ADH and ADH-NAD$^+$ complex in the enhancing of pressure and temperature respectively.

The values of Michaelis-Menten constants, K_M, the maximum velocity, v_m and maximum rate constant, k_m listed in Table 3 were evaluated from Eq(4) and (5) by using Figure 1 and 2 respectively. K_M values decrease with increasing pressure and temperature like the overall equilibrium constant, K of Eq(8) which behaviour will be explained later. The values of v_m and k_m shown in Table 3 increase with elevating pressure and temperature in the same way that v and k_o do.

Using the v_m and k_m values in Table 3 evaluated from Eq(4) and (5) by plotting Figure 1 and 2, we obtained the values of the all rate constants of Eq(1) to list them in Table 4. The values of k_{-1} and k_{-2} were calculated from the K_1 and K_2 using the k_1 and k_2 values calculated from the intercept and slope of Figure 3 by using Eq(7). These all kinds of rate constants increasing temperature and pressure like k_o and k_m mentioned above but only k_{-1} value has a maximum values at 600 bars at the lowest concentration of ethylalcohol, S_1 and decrease with elevating pressure.

The K_1, K_2 and K values of Eq(8) were calculated from Eq(9) by using the data of Table 4 to be shown in Table 5. K values decrease with enhancing temperature and pressure as K_2 values but K_1 values increase with enhancing temperature and pressure. The reason for K_1 behavior is considered to be caused by the fact that the segmental motion of ADH macromolecule is elevated by increasing temperature. The

Table 2. Observed Rates (v× 10^4, mol·dm^{-3}sec^{-1}) and Rate Constants (k_0 × 10^3, sec^{-1}) of the Dehydrogenation of Ethylalcohol Catalyzed by ADH-NAD$^+$ [a]

Conc. of EOH(M) press. (bars)			S_1	S_2	S_3	S_4	S_5
25 °C	1	v	5.01	6.15	7.82	9.85	10.8
			(±0.13)	(±0.25)	(±0.37)	(±0.26)	(±0.54)
		k_0	1.01	1.22	1.54	1.91	2.07
			(±0.06)	(±0.05)	(±0.07)	(±0.05)	(±0.10)
	300	v	5.55	6.68	8.52	10.7	11.8
			(±0.47)	(±0.51)	(±0.05)	(±0.09)	(±0.72)
		v_0	1.18	1.40	1.76	2.15	2.30
			(±0.18)	(±0.15)	(±0.01)	(±0.21)	(±0.15)
	600	v	6.13	7.36	8.84	8.98	12.3
			(±0.59)	(±0.72)	(±0.16)	(±0.17)	(±0.72)
		k_0	1.35	1.61	1.89	2.39	2.53
			(±0.13)	(±0.26)	(±0.29)	(±0.17)	(±0.12)
	900	v	6.86	7.97	8.89	11.9	12.8
			(±0.42)	(±0.62)	(±0.83)	(±0.76)	(±0.67)
		k_0	1.49	1.75	2.13	2.56	2.70
			(±0.30)	(±0.34)	(±0.21)	(±0.17)	(±0.13)
	1200	v	7.82	9.24	10.9	13.3	13.9
			(±0.69)	(±0.92)	(±0.44)	(±0.31)	(±0.74)
		k_0	1.77	2.06	2.41	2.91	3.02
			(±0.38)	(±0.43)	(±0.31)	(±0.21)	(±0.28)
	1500	v	10.1	11.4	13.8	15.8	16.1
			(±0.28)	(±0.43)	(±0.56)	(±0.37)	(±0.86)
		k_0	2.30	2.58	2.99	3.45	3.51
			(±0.05)	(±0.10)	(±0.05)	(±0.11)	(±0.18)
35 °C	1	v	6.78	7.91	9.50	11.3	12.3
			(±0.44)	(±0.33)	(±0.61)	(±0.55)	(±0.88)
		k_0	1.47	1.69	2.01	2.33	2.57
			(±0.09)	(±0.07)	(±0.13)	(±0.11)	(±0.19)
	300	v	7.54	8.59	10.3	12.2	13.1
			(±0.59)	(±0.77)	(±0.24)	(±0.65)	(±0.71)
		k_0	1.65	1.86	2.22	2.61	2.74
			(±0.14)	(±0.17)	(±0.17)	(±0.15)	(±0.21)
	600	v	8.29	9.48	11.4	13.2	13.9
			(±0.18)	(±0.25)	(±0.31)	(±0.10)	(±0.20)
		k_0	1.81	2.10	2.48	2.79	2.94
			(±0.11)	(±0.09)	(±0.10)	(±0.21)	(±0.16)
	900	v	8.56	10.4	12.2	14.0	15.2
			(±0.72)	(±0.95)	(±0.17)	(±0.23)	(±0.36)
		k_0	1.93	2.32	2.66	2.98	3.14
			(±0.21)	(±0.20)	(±0.23)	(±0.16)	(±0.19)
	1200	v	9.53	11.4	13.4	14.9	16.5
			(±0.17)	(±0.36)	(±0.49)	(±0.24)	(±0.90)
		k_0	2.19	2.59	2.97	3.29	3.51
			(±0.02)	(±0.19)	(±0.24)	(±0.28)	(±0.20)
	1500	v	9.71	13.3	14.9	16.9	18.1
			(±0.59)	(±0.27)	(±0.23)	(±0.88)	(±0.49)
		k_0	2.25	3.04	3.36	3.75	3.91
			(±0.21)	(±0.28)	(±0.11)	(±0.16)	(±0.08)

[a] For details, see Experimental procedures. Values of v and k_o were evaluated as the average value calculated by using Eq(2) and (3) from three measurements.
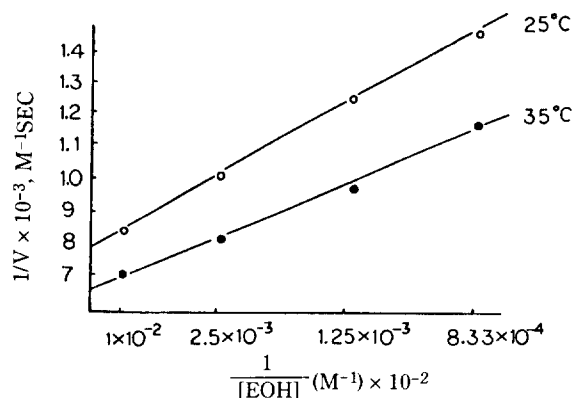
Figure 1. Lineweaver Burk plots of thde dehydrogenation of ethyl-alcohol using Eq(4). From the intercept and slope of the plot, maximum velocity, v_m, Michaelis-Menten constant, K_M were obtained respectively. Correlation coefficients of the plot were $r = 0.992$ for 25 °C(○) and $r = 0.997$ for 35 °C(●) at 900 bars respectively.
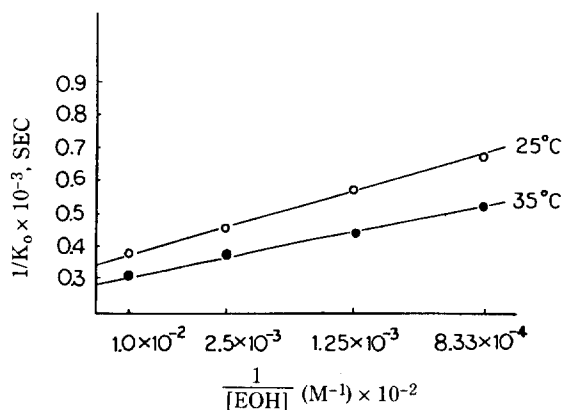

Figure 2. A new type of Lineweaver-Burk plot of the dehydrogenation of ethylalcohol by using Eq(5). From the intercept and slope of the plot, maximum rate constant, k_m and Michaelis-Menten constant, K_M were calculated respectively. Correlation coefficients were the same, $r = 0.992$ for 25 °C(○) and $r = 0.996$ for 35 °C(●) at 900 bars respectively.

elevated segmental motion of ADH molecule make NAD^+ and EOH molecules diffuse easily into a hydrophobic pocket formed at the junction of catalytic binding domain of ADH. In addition, a driving force making NAD^+ and EOH molecules go into the pocket is increased with elevating pressure. Consequently, temperature and pressure help a binary and ternary complex of Eq(1) and (8) be made.
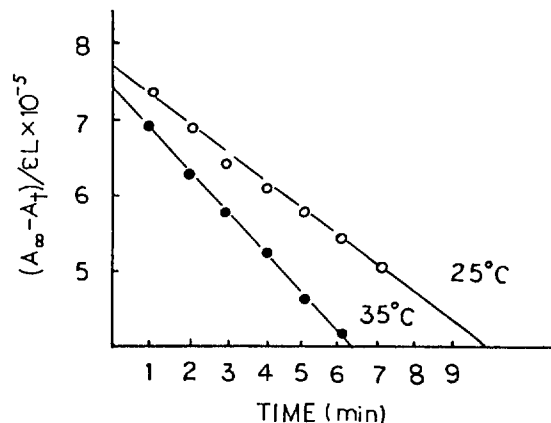

Figure 3. Determination of rate constant k_1 and k_2 using Eq(7). The molar extinction coefficient of NADH was 6220 $mol^{-1}dm^3cm^{-1}$ From the slope of the plot, the rate constant, k_1 and k_2 for the forward reaction of the first and second step of the dehydrogenation of ethylalcohol were evaluated. Correlation coefficients were $r = 0.998$ for 25 °C(○) and 35 °C(●) at 900 bars respectively.

As shown in Table 6, the enthalpy at the first step of Eq(8), ΔH_1° are positive and decrease with enhancing with increasing pressure but the other enthalpy ΔH_M°, ΔH° and ΔH_2° are all negative, and increase with enhancing pressure. All activation enthalpy decrease with elevating pressure. This fact means that the stability of the activated ternary complex increase with enhancing pressure. Plotting these all entropies versus reaction coordinate at 900 bars, we had a typical enthalpy profiles as shown in Figure 4.

From Table 6 and 7, we could known that the first step-kinetic is controlled by enthalpy but the second step-kinetic state is controlled by entropy, therefore the over all kinetic state is controlled by enthalpy as shown in Table 4.

As listed in Table 7, all entropies except the first step entropies ΔS_1° of Eq(1) are all negative, and the absolute value of all entropy decrease with increasing pressure. Both enthalpy and entropy of the first step are positive and their values decrease with enhancing pressure. It means that the first step of Eq(8) is endothermic reaction and endothermic strength decrease with elevating pressure. On the contrary, the enthalpy and entropy of the second step are negative and their absolute values decrease with elevating pressure. It means that the second step is exothermic reaction and results in a decrease in order since the entropy is a measure of the disorder of a system. The degree of exothermic reaction and disorder with increasing pressure. At the same time, all activation entropies but ΔS_{-2}^{*} at 1 bars have small positive

Table 3. Michaelis-Menten Constant ($K_M \times 10^3$, mol·dm^{-3}) and Maximim Rates and Rate Constants ($v_m \times 10^3$, mol·dm^{-3}sec^{-1} and $k_m \times 10^3$, sec^{-1}) of the Dehydrogenation of Ethylalcohol Catalyzed by ADH-NAD$^+$ [a]

Press. (bars)		1	300	600	900	1200	1500
	K_M	0.97 ± 0.07	0.96 ± 0.05	0.87 ± 0.06	0.72 ± 0.06	0.65 ± 0.02	0.51 ± 0.03
25 °C	v_m	1.08 ± 0.08	1.18 ± 0.06	1.23 ± 0.09	1.28 ± 0.06	1.39 ± 0.10	1.62 ± 0.02
	k_m	2.07 ± 0.17	2.29 ± 0.11	2.53 ± 0.14	2.69 ± 0.17	3.02 ± 0.21	3.51 ± 0.27
	K_M	0.68 ± 0.05	0.59 ± 0.01	0.58 ± 0.05	0.57 ± 0.04	0.56 ± 0.04	0.47 ± 0.04
35 °C	v_m	1.23 ± 0.09	1.29 ± 0.11	1.39 ± 0.05	1.52 ± 0.11	1.66 ± 0.13	1.81 ± 0.17
	k_m	2.57 ± 0.18	2.74 ± 0.23	2.94 ± 0.18	3.13 ± 0.25	3.51 ± 0.31	3.91 ± 0.27

[a] K_M, v_m and k_m values were calculated from Eq(4) and (5) by using Figure 1 and 2.

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Table 4. Rate Constants (k_1 and $k_{-1} \times 10^2$, mol dm^{-3}sec^{-1}, and k_2 and $k_{-2} \times 10^3$, mol·dm^{-3}sec^{-1}) of the Dehydrogenation of Ethylalcohol Catalyzed by ADH-NAD$^+$ [a]

25 °C

		1	300	600	900	1200	1500
S_1	k_1	61.1 (±1.22)	69.4 (±2.36)	89.1 (±2.14)	93.1 (±3.14)	107 (±1.29)	128 (±1.45)
	k_{-1}	5.85 (±0.03)	5.44 (±0.04)	6.38 (±0.01)	6.20 (±0.04)	6.22 (±0.06)	5.55 (±0.03)
	k_2	0.50 (±0.01)	0.55 (±0.03)	0.73 (±0.03)	0.79 (±0.05)	0.92 (±0.07)	1.09 (±0.09)
	k_{-2}	1.23 (±0.06)	2.76 (±0.02)	5.49 (±0.04)	6.87 (±0.05)	11.9 (±0.03)	20.1 (±0.13)
S_2	k_1	47.2 (±2.32)	53.5 (±3.26)	66.2 (±4.11)	71.5 (±3.29)	79.7 (±5.11)	93.1 (±4.86)
	k_{-1}	4.07 (±0.03)	4.32 (±0.04)	4.72 (±0.03)	4.73 (±0.01)	4.59 (±0.02)	3.95 (±0.03)
	k_2	0.59 (±0.04)	0.64 (±0.02)	0.83 (±0.04)	0.92 (±0.07)	1.05 (±0.02)	1.20 (±0.01)
	k_{-2}	1.72 (±0.09)	4.94 (±0.24)	9.61 (±0.62)	13.2 (±0.72)	17.2 (±0.49)	30.6 (±0.36)
S_3	k_1	28.4 (±1.72)	32.4 (±3.11)	37.3 (±2.47)	42.6 (±1.69)	45.9 (±1.98)	50.7 (±2.52)
	k_{-1}	2.41 (±0.04)	2.47 (±0.01)	2.60 (±0.06)	2.76 (±0.03)	2.58 (±0.01)	2.08 (±0.01)
	k_2	0.72 (±0.05)	0.79 (±0.06)	0.95 (±0.07)	1.13 (±0.09)	1.21 (±0.11)	1.35 (±0.03)
	k_{-2}	2.69 (±0.02)	8.80 (±0.42)	22.8 (±0.72)	30.3 (±0.11)	41.6 (±0.18)	56.0 (±0.47)
S_4	k_1	8.51 (±0.11)	9.29 (±0.16)	10.93 (±0.15)	11.9 (±0.72)	12.3 (±0.17)	13.8 (±0.67)
	k_{-1}	0.65 (±0.01)	0.67 (±0.05)	0.68 (±0.01)	0.63 (±0.06)	0.59 (±0.04)	0.47 (±0.01)
	k_2	0.89 (±0.04)	0.94 (±0.06)	1.15 (±0.17)	1.20 (±0.49)	1.34 (±0.53)	1.52 (±0.48)
	k_{-2}	16.4 (±0.72)	31.7 (±0.36)	103 (±0.14)	163 (±0.19)	118 (±2.3)	384 (±0.99)
S_5	k_1	0.89 (±0.04)	0.98 (±0.03)	1.25 (±0.13)	1.31 (±0.36)	1.24 (±0.11)	1.34 (±0.13)
	k_{-1}	5.67 (±0.42)	4.98 (±0.09)	5.35 (±0.09)	5.14 (±0.09)	5.45 (±0.06)	4.39 (±0.03)
	k_2	0.97 (±0.01)	1.05 (±0.14)	1.35 (±0.06)	1.36 (±0.14)	1.37 (±0.13)	1.52 (±0.14)
	k_{-2}	92.1 (±1.36)	84.5 (±2.49)	109 (±2.15)	115 (±4.25)	125 (±6.72)	127 (±3.69)

35 °C

		1	300	600	900	1200	1500
S_1	k_2	0.78 (±0.01)	0.80 (±0.02)	0.93 (±0.03)	0.93 (±0.05)	1.03 (±0.04)	1.07 (±0.08)
	k_{-2}	7.04 (±0.05)	6.26 (±0.04)	11.0 (±0.02)	13.1 (±0.03)	22.1 (±0.13)	25.8 (±0.12)
	k_1	69.4 (±2.34)	74.3 (±2.15)	82.1 (±4.07)	85.7 (±3.29)	89.8 (±5.14)	101 (±6.42)
S_2	k_{-1}	4.25 (±0.04)	3.81 (±0.01)	3.97 (±0.03)	3.79 (±0.01)	3.86 (±0.13)	3.45 (±0.03)
	k_2	0.90 (±0.02)	0.97 (±0.04)	1.06 (±0.06)	1.08 (±0.04)	1.12 (±0.03)	1.30 (±0.07)
	k_{-2}	12.8 (±0.72)	14.5 (±0.49)	20.1 (±0.54)	27.7 (±0.34)	34.3 (±0.29)	43.9 (±0.39)
	k_1	36.1 (±1.36)	41.6 (±3.24)	46.0 (±2.79)	46.8 (±4.21)	50.1 (±2.92)	54.2 (±3.11)
S_3	k_{-1}	2.16 (±0.03)	2.38 (±0.06)	2.16 (±0.01)	2.01 (±0.09)	2.09 (±0.03)	1.76 (±0.02)
	k_2	0.94 (±0.05)	1.13 (±0.09)	1.21 (±0.03)	1.25 (±0.07)	1.29 (±0.10)	1.42 (±0.02)
	k_{-2}	16.7 (±0.13)	31.0 (±0.24)	38.1 (±0.39)	48.6 (±2.3)	74.7 (±1.25)	74.8 (±0.49)
	k_1	10.7 (±0.14)	11.6 (±0.16)	12.3 (±0.73)	12.6 (±0.24)	13.3 (±0.65)	14.3 (±0.55)
S_4	k_{-1}	0.55 (±0.01)	0.57 (±0.02)	0.48 (±0.01)	0.44 (±0.09)	0.43 (±0.04)	0.34 (±0.02)
	k_2	1.14 (±0.04)	1.27 (±0.02)	1.33 (±0.03)	1.34 (±0.02)	1.41 (±0.05)	1.51 (±0.04)
	k_{-2}	14.0 (±0.91)	125 (±0.42)	111 (±0.51)	131 (±0.43)	343 (±0.63)	424 (±0.92)
	k_1	11.5 (±0.13)	12.0 (±1.21)	12.1 (±0.86)	13.1 (±0.54)	13.9 (±0.91)	14.7 (±1.36)
S_5	k_{-1}						
	k_2	1.25 (±0.02)	1.33 (±0.15)	1.38 (±0.09)	1.41 (±0.13)	1.52 (±0.13)	1.64 (±0.9)
	k_{-2}						

[a] The k_1 and k_2 values were evaluated from Figure 3 by using Eq(7). Values of k_{-1} were calculated from substitution of k_1 and k_2 values into $K_M = (k_{-1} + k_2)/k_1$.

values. This fact means that the reaction of Eq(1) has a entropy reaction which will be mentioned in detail later because a role of absolute temperature-entropy product, $T\Delta S^*$ is larger that of enthalpy, ΔH^* in a activation free energy, ΔG^*. Besides it suggest that not only a ternary complex has a more ordered structure, but also water molecules make a iceburg close by the activated complex at the transition state.

A volume and activation volume calculated from Eq(10)

Table 5. Equilibrium Constants (K, K_1 and K_2) of the Dehydrogenation of Ethylalcohol Catalyzed by ADH-NAD$^+$ [a]

Press. (bars)		1	300	600	900	1200	1500
25 °C	K	4.23 ± 0.12	2.54 ± 0.17	1.84 ± 0.09	1.73 ± 0.11	1.33 ± 0.07	1.27 ± 0.08
	K_1	10.4 ± 0.43	12.7 ± 0.62	13.9 ± 0.94	15.0 ± 0.59	17.2 ± 1.24	23.3 ± 1.18
	K_2	0.41 ± 0.001	0.19 ± 0.001	0.13 ± 0.001	0.11 ± 0.001	0.07 ± 0.002	0.05 ± 0.004
35 °C	K	1.81 ± 0.05	1.98 ± 0.04	1.73 ± 0.07	1.60 ± 0.06	1.08 ± 0.11	1.22 ± 0.12
	K_2	16.2 ± 0.62	16.9 ± 0.92	20.5 ± 0.42	22.4 ± 0.37	23.1 ± 0.25	29.2 ± 0.14
	K_2	0.11 ± 0.001	0.15 ± 0.0001	0.08 ± 0.004	0.07 ± 0.003	0.04 ± 0.001	0.04 ± 0.002

[a] Overall equilibrium constants K's were obtained from Eq(9) and the first and second step equilibrium constants, K_1 and K_2 from $K = K_1K_2 = (k_1/k_{-1})(k_2/k_{-2})$ by using the data shown in Table 4.
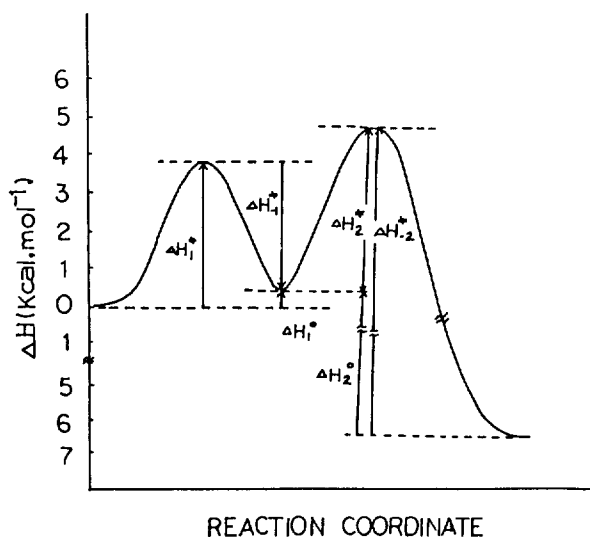
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Figure 4. Enthalpy profile of the dehydrogenation of ethylalcohol. The profile was consisted of enthalpy data at 900 bars listed in Table 6.
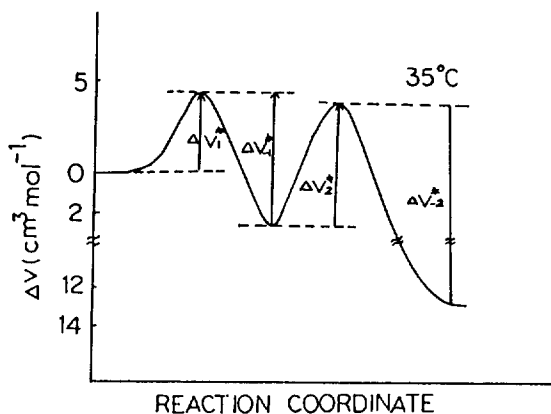

REACTION COORDINATE

Figure 5. Volume profile of the dehydrogenation of ethylalcohol. The profile was consisted of volume data at 35 °C listed in Table 8.

and (11) were listed in Table 8 and shown at Figure 5, typically. The $\Delta V_1{}^o$ has negative value but $\Delta V_2{}^o$ has positive values. It means that the volume of the reaction system of Eq(8) is shrunk when the ternary complex is formed, and en-

Table 6. Various Enthalpies for the Dehydrogenation of Ethylalcohol Catalyzed by ADH-NAD⁺ at 35°C[a]

Enthalpy (kcal mol⁻¹) Press. (bars)	ΔH^o_M	ΔH^o	ΔH^o_1	ΔH^o_2	ΔH^o_m	ΔH^*_1	ΔH^*_{-1}	ΔH^*_2	ΔH^*_{-2}
1	-6.38 (±0.18)	-15.7 (±0.37)	8.06 (±0.20)	-23.8 (±0.59)	3.96 (±0.24)	11.5 (±0.28)	3.49 (±0.19)	8.21 (±0.21)	31.8 (±1.79)
300	-5.13 (±0.32)	-4.16 (±0.29)	5.22 (±0.24)	-9.14 (±0.61)	3.28 (±0.21)	3.61 (±0.24)	-1.60 (±0.11)	5.12 (±0.42)	14.95 (±1.82)
600	-6.94 (±0.29)	-1.23 (±0.11)	7.05 (±0.29)	-8.30 (±0.42)	2.76 (±0.11)	3.83 (±0.19)	-3.24 (±0.14)	4.50 (±0.17)	12.80 (±1.18)
900	-7.21 (±0.52)	-1.45 (±0.09)	7.32 (±0.21)	-8.73 (±0.37)	2.76 (±0.19)	3.88 (±0.11)	-3.43 (±0.29)	3.06 (±0.18)	11.82 (±0.92)
1200	-5.26 (±0.48)	-3.83 (±0.13)	5.33 (±0.31)	-9.21 (±0.44)	2.73 (±0.21)	2.92 (±0.21)	-2.42 (±0.19)	2.15 (±0.16)	11.34 (±1.10)
1500	-4.01 (±0.21)	-0.78 (±0.11)	4.09 (±0.17)	-4.94 (±0.19)	1.96 (±0.32)	-0.04 (±0.01)	-4.30 (±0.17)	-0.34 (±0.001)	4.56 (±0.42)

[a]Values of enthalpies and activation enthalpies were obtained from Eq(10) and (11) at 35 °C by using the data in Table 4 and 5. All subscripts agree with the subscripts of Michaelis-Menten constant of equilibrium and rate constants shown in Eq(1) and (8).

Table 7. Various Entropies for the Ethylalcohol Dehydrogenation Catalyzed by ADH-NAD⁺ at 35°C[a]

Entropy (cal mol⁻¹k⁻¹) Press. (bars)	ΔS^o_M	ΔS^o	ΔS^o_1	ΔS^o_2	ΔS^*_m	ΔS^*_1	ΔS^*_{-1}	ΔS^*_2	ΔS^*_{-2}
1	-92.3 (±5.53)	-49.5 (±1.24)	31.7 (±0.71)	-81.2 (±2.03)	-56.1 (±3.36)	-20.2 (±0.50)	-51.6 (±2.90)	-44.6 (±1.11)	36.2 (±2.04)
300	-90.3 (±4.78)	-13.6 (±1.01)	32.2 (±714)	-34.0 (±1.42)	-60.0 (±3.62)	-47.4 (±3.92)	-70.0 (±6.11)	-56.6 (±1.89)	-20.4 (±1.96)
600	-96.5 (±5.52)	-2.9 (±0.14)	28.9 (±0.46)	-31.9 (±1.62)	-61.4 (±4.18)	-46.1 (±2.48)	-75.1 (±3.96)	-58.1 (±1.24)	-26.2 (±2.11)
900	-97.5 (±5.67)	-3.8 (±0.21)	30.0 (±0.72)	-33.6 (±1.49)	-61.3 (±5.14)	-45.9 (±1.69)	-75.8 (±4.28)	-62.8 (±2.56)	-29.1 (±2.14)
1200	-91.2 (±6.12)	-12.3 (±0.47)	23.6 (±1.01)	-36.0 (±3.2)	-61.2 (±4.96)	-48.9 (±4.21)	-72.4 (±6.14)	-65.5 (±4.96)	-29.6 (±1.98)
1500	-87.7 (±4.98)	-2.1 (±0.19)	20.0 (±0.28)	-22.4 (±2.14)	-63.5 (±5.11)	-58.4 (±3.64)	-79.0 (±4.14)	-73.5 (±6.38)	-85.6 (±3.69)

[a]Values of entropies and activation entropies were evaluated from Eq(10) and (11) at 35 °C by using the data in Table 4 and 5. All subscripts agree with the subscripts of Michaelis-Menten constants of equilibrium and rate constants shown in Eq(1) and (8).

Table 8. Various Volumes for the Ethylalcohol Dehydrogenation Catalyzed by ADH-NAD+ ᵃ

Volume (cm³/mol) Temp.(°C)	ΔV_M^o	ΔV^o	ΔV_1^o	ΔV_2^o	$\Delta V_m^{\ddagger}$	$\Delta V_1^{\ddagger}$	$\Delta V_{-1}^{\ddagger}$	$\Delta V_2^{\ddagger}$	$\Delta V_{-2}^{\ddagger}$
25 °C	8.15 (±0.92)	14.46 (±1.42)	−9.58 (±0.45)	13.65 (±1.14)	−9.29 (±0.49)	−10.11 (±0.96)	4.73 (±0.04)	−13.2 (±0.44)	−40.5 (±2.65)
35 °C	4.72 (±0.42)	12.52 (±0.96)	−12.52 (±1.14)	13.61 (±1.11)	−7.08 (±0.04)	−4.34 (±0.19)	3.37 (±0.24)	−6.11 (±0.34)	−17.38 (±1.96)

ᵃValues of volume and activation volume were calculated from Eq(10) and (11) by using the data in Table 4 and 5. All subscripts agree with the subscripts of Michaelis-Menten constant of equilibrium and rate constants shown in Eq(1) and (8).
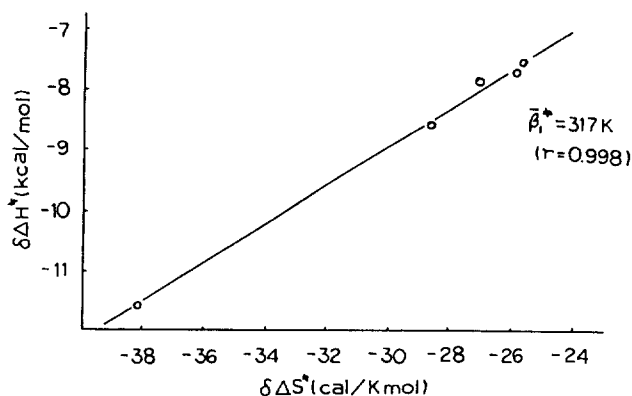

Figure 6. The isokinetic temperature for the first step of the ethylalcohol dehydrogenation catalyzed by ADH-NAD⁺. This plot was made of enthalpy and entropy change for changing pressure at 35 °C as shown Table 6 and 7.
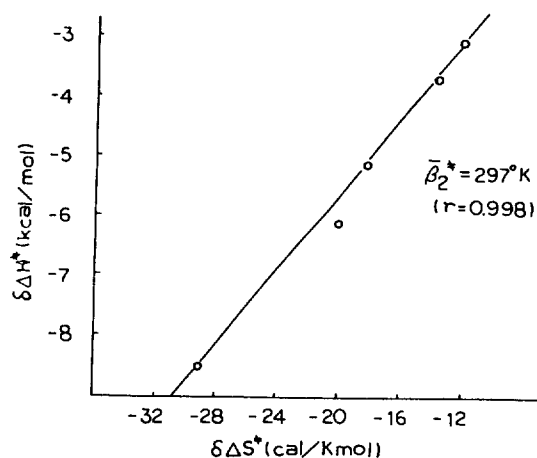
Figure 7. The isokinetic temperature for the second step of the ethylalcohol dehydrogenation catalyzed by ADH-NAD⁺. This plot was made of enthalpy and entropy change for changing pressure at 35 °C as shown Table 6 and 7.

larged when the complex is changed into product. The values of $\Delta V_1^{\ddagger}$ and $\Delta V_2^{\ddagger}$ are negative and their absolute values decrease when temperature of the reaction system of Eq(1) is increased from 25 °C to 35 °C. This fact explains that a lot of water molecules are bound to the enolgated activated complexes at the first and second transition states and weakly bound water molecules get out when temperature is elevated.

Finally, to determine in details whether the reaction of

Table 9. Isoequilibrium Temperatures($\bar{\beta}$) and Isokinetic Temperatures($\bar{\beta}$*) of the Dehydrogenation of Ethylalcohol Catalyzed by ADH-NAD= at 900 Barsᵃ

$\bar{\beta}$ (K) Temp.(°C)	$\bar{\beta}^o$	$\bar{\beta}_1^o$	$\bar{\beta}_2^o$	$\bar{\beta}_1^{\ddagger}$	$\bar{\beta}_{-1}^{\ddagger}$	$\bar{\beta}_2^{\ddagger}$	$\bar{\beta}_{-2}^{\ddagger}$
35 °C	309	324	339	317	300	297	387

ᵃThe values of all $\bar{\beta}$ and $\bar{\beta}$* were calculated from a plot similar to Figure 6 by using Eq(12) and (13) respectively. The subscripts of $\bar{\beta}$ and $\bar{\beta}$* agree with the subscripts respectively. Correlation coefficients of all $\bar{\beta}$ and $\bar{\beta}$* were greater than 0.99.

Eq(1) is controlled by enthalpy or entropy we obtained isoequilibrium temperature, $\bar{\beta}$ and isokinetic temperature, β* from a slope of Figure 6 and 7 plotted by using Eq(12) and (13). The $\bar{\beta}$ and β* values were written in Table 9. Generally, when $\bar{\beta}$ and β* are higher than experimental temperature, 35 °C we say equilibrium and kinetics are controlled by enthalpy. But if their values are lower than experimental temperature, we call the equilibrium and kinetics are controlled by entropy since the contribution to free energy, ΔG and activation free energy, $\Delta G^{\ddagger}$ from entropy, ΔS and activation entropy $\Delta S^{\ddagger}$ than that from enthalpy, ΔH and activation enthalpy, $\Delta H^{\ddagger}$ respectively. Accordingly from Table 9 we could suggest the equilibrium of Eq(9) for over all, the first step and second step are controlled by enthalpy, and kinetics at the first step is controlled by enthalpy but the kinetics at the second step is controlled by entropy. This work was supported by the Basic Research Program, Ministry of Education, Korea, 1987.

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Photoaddition Reactions of Alkynes to Quinonoid Compounds

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Received August 29, 1988

UV irradiation of anthraquinone and diphenylacetylene in benzene gave 1:1 photoadduct (**7**) and cyclization product (**8**). The photoreaction of anthrone and diphenylacetylene in dichloromethane afforded the photooxidation products (**7, 8**, and **9**) in air. The photoproduct (**7**) underwent the cyclization reaction during the purification by the column chromatography (silica gel). When irradiated with 350 nm UV light, the product (**11**) of benzil reacted with diphenylacetylene to give a photoadduct (**12**).

Introduction

The photochemical addition reactions of *p*-quinones to compounds containing olefinic or acetylenic linkages have been the subject of a number of publications.[1-11] Photodimerization of *p*-benzoquinone gives either cyclobutanes or spirooxetanes depending on the nature of the quinone.[1-4] The photoaddition of diphenylacetylene to *p*-benzoquinone forms a quinone methide(**1**), probably through an unstable intermediate, spiro-oxetene(**2**). In contrast to *p*-benzoquinone, methoxy-*p*-benzoquinone gives the cyclobutene.[4]

Scheme 1

We recently reported that chloranil readily forms a novel photorearrangement product by the photoreaction with some cyclic olefins.[12] Irradiation of the quinonoid compounds and

diphenylacetylene in dichloromethane gives the photoproducts, such as (**3**), (**4**), or (**5**).[1-8,13] When irradiated with 350 nm UV light, cyclohexene reacts with thioxanthone, anthrone, and anthraquinone, to give 1:1 photoadducts, such as (**6**).[13] These spiropoxetanes decompose to the starting materials by 254 nm UV light.[13]

In connection with our continuous studies toward the photoaddition reactions of *p*-quinone derivatives with alkynes, we now report that irradiation of the quinonoid compounds and diphenylacetylene gives not only 1:1 photoaddut (**7**), but also the photocyclization product(**8**) of (**7**).

The purpose of this paper is to describe the photochemical formation of the polynuclear aromatic compounds from the quinonoid compounds. It is also worth to notice that the photooxidation products are formed by the photoreaction of anthrone and diphenylacetylene in air.

Experimental

Materials. Dichloromethane, *n*-hexane, ethylacetate, chloroform, and benzene were purified before use. Diphenylacetylene, anthrone, anthraquinone, and benzil were purchased from Aldrich Chemical Company and used as received. The column chromatography was performed by using Kiesel gel (Merck Co., 70-230 mesh). Kiesel gel 60 F$_{254}$ (Merck Co., silica gel) was used for the thin layer chromatography.

Instruments. Infrared spectra were recorded on a Perkin-Elmer 283B Grating Spectrophotometer in KBr pellets. ^{1}H-NMR spectra were obtained on a Bruker AC-100 Spec-