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THE CALORIMETRIC ANALYSIS OF UREA-UREASE SYSTEM

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In the present experiment the temperature change in the reaction system with time was measured in order to investigate the reaction rate continuously and the reaction heats of the urea-urease reaction by using the calorimeter.

The heats of reaction were endothermic theoretically, but NH_3 and CO_2 produced as result of the reaction generated the heats such as heats of dissolution and heat of neutralization.

Then the reaction was exothermic in resultant. It was found that the Michaelis-Menten law was obeyed accurately up to a certain concentration of urea, but at high concentration of it there was some falling-off of the rate as in the former experiment.

The Michaelis constants were also determined by using the Dixon's method.

Introduction

Nearly all of the measurements of the urea-urease reaction have been carried out in the presence of a buffer by the method of analytical determination of NH_3 produced as result of the reaction with time. The present author has investigated the thermal nature of the enzymic reactions in the presence of buffer and in the absence of buffer, *i. e.*, in an aqueous solution, by the thermo-analytical method[1] by which the rate can be measured continuously and also the reaction heat can be measured. Urease-catalyzed hydrolysis of urea follows the stoichiometric equation,

$$CO(NH_2)_2(aq) + H_2O = 2NH_3(g) + CO_2(g) + aq.$$

The reaction heat which is calculated by the author from the resultant of each heat of formation between the reaction system and the product system is endothermic, *i. e.*, $\Delta H = 27.81$ kcal/M. Ammonia and carbon dioxide produced as result of the reaction, however, evolve the heats of dissolution and neutralization in the reaction solution exothermically, which are 32.31 kcal in total, as calculated from the chemical table. Therefore, the heat evolved in the reaction system shows practically the difference of these heats of the above reactions. It is 4.50 kcal/M, and the present author can find that this value coincides nearly with the heat of reaction calculated from the experimental data of thermal analysis of the reaction in the absence of buffer, *i. e.*, the reaction in an aqueous solution.

It may be observed from this result that the intermediate compounds such as carbamic acid

1) T. Kôsaki, *This Journal*, **9**, 63 (1935).
E. Suito, *ibid.*, **13**, 74 (1939).
J. Osugi and K. Hiromi, *ibid.*, **22**, 76 (1952)

and carbamate in the first step of the reaction are not produced, and urea decomposes instantly into NH_3 and CO_2 in the reaction solution.

On the other hand, the following work is undertaken in order to investigate the applicability of the Michaelis-Menten law, and to obtain the Michaelis constants. The reaction rate, dx/dt, is obtained from both the Conway's method and the indophenol reagent method modified by Hatano[2]. This rate is directly proportional to the temperature change with time, dT/dt, measured by thermal analysis. The present author then examined an applicability of the Michaelis-Menten law by the relation between initial parts of dT/dt curve and urea concentrations. The Michaelis-Menten law in lower concentration range is verified to be obeyed accurately up to a urea concentration of 4×10^{-1} M/L in the presence of buffer and of 10^{-1} M/L in the absence of buffer, but at higher concentrations there is some falling-off of the rate. Namely at higher concentrations the curve reaches maximum, and then decreases.

The Michaelis constants can be obtained by plotting the reciprocals of rates versus the reciprocals of urea concentrations by the Dixon's method[3]. The values obtained in this way are 6.3×10^{-2} in the presence of buffer and 3.2×10^{-2} in the absence of buffer, *i. e.*, in an aqueous solution.

Experimentals

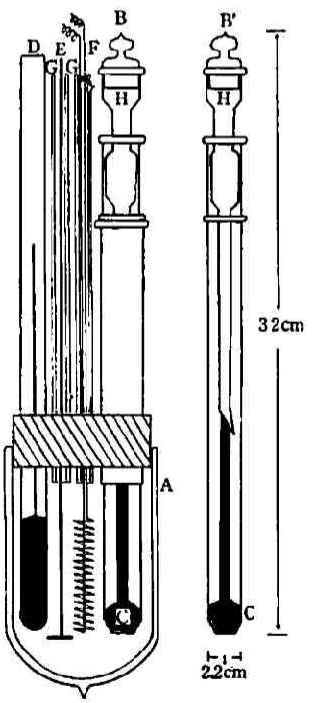

Fig. 1 Calorimeter

2) H. Hatano and T. Kirita, *J. Japanese Chem., Special ser.*, **34**, 41 (1958)
3) M. Dixon, *Biochem. J.*, **55**, 170 (1953)
H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934)

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Apparatus The measurement of the temperature change of the reaction system is performed by means of a glass calorimeter, as shown in Fig. 1.

(A): Silver plated glass Dewar vessel containing 140ml of substrate solution

(B): Glass tube with glass stop cock (C) which contains 10ml of enzyme solution

(B'): Illustration of (B) as explanatory diagram

(H): Piston rod which is used to open the stop cock (C), and to draw out the enzyme solution into the reaction vessel

(D): Beckmann's thermometer (0.001 or 0.01°C)

(E): Glass stirrer which moves 34 times/min, up and down. The influence of stirring has no effect on the temperature of the reaction system

(F): Heater with a platinum wire resistance movable up and down. The substrate solution in (A) was heated to such the same temperature as that of the reaction, and then the heater is drawn up to the surface of the solution

(G): Vacuum glass tube with which the thermal effect is avoided from outside.

The whole apparatus is dipped in a thermostat at 40°±0.01°C. The reaction temperature in the vessel can be held at 40°±0.001°C throughout two hours.

Materials Urea: Takeda's guaranteed reagent

Urease: Crystalline urease prepared by Uezu's method[4] was kindly provided in the Biochemical Laboratory of Kyoto University, and σ urease was imported from the Sigma Chemical Company in U. S. A. for jack beans. The crystalline enzyme is dissolved with 10ml distilled water or with 10ml phosphate buffered solution of pH=6.7 and pH=7.0. The solution dissolved is frozen with dry ice at once and stored in a Dewar vessel filled with dry ice as stock solution.

Buffer solution: KH_2PO_4 and $Na_2HPO_4 \cdot 2aq$, both Merk's guaranteed reagents

Other reagent: Takeda's guaranteed reagents

Distilled water: Distilled water produced in all glass made apparatus

The concentrations of urea solution, which are prepared at 20°C, are 10^{-3}, 10^{-2}, 10^{-1}, 0.4, and

Table 1 Concentrations and activities of enzymes used

Enzyme	Solvent	Specific Activity units/g. protein	pH	Concentration mg/10 ml	Activity units/mg. protein
U_4	Buffer	80,000	6.7	12.9	1032.0
U_5	〃	〃	〃	16.8	1344.0
U_6	〃	85,000	7.0	26.3	2335.5
U_7	〃	65,000	6.7	21.9	1423.5
U_8	〃	〃	〃	28.5	1852.5
U_9	Distilled water	68,000	5.54–5.95	29.4	1999.2
U_{10}	Buffer	67,000	6.7	59.7	4009.9
$\frac{1}{2}U_5$	〃	80,000	〃	8.4	672.0
$\frac{1}{2}U_6$	〃	85,000	7.0	13.15	1167.75
$\frac{3}{10}U_5$	〃	80,000	6.7	5.04	403.2

4) Uezu and K. Obashi, *Biol. Chem. J.*, **31**, (8). 715 (1959)

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1 M/L respectively and the enzyme concentrations and activities used are as indicated in Table 1. σ-urease is used in the concentration of 0.09 g/10 ml.

Procedure After the whole apparatus was dipped in a thermostat at 40°±0.01°C, the urea solution 140 ml of a certain concentration, is heated in the reaction vessel (A), to such the same temperature as that of reaction. The frozen enzyme solution of 10 ml is dissolved at room temperature spontaneously. The glass vessel (B) which contains this dissolved enzyme solution of 10 ml is immersed in the thermostat at 40°±0.01°C for 15 minutes set on the reaction vessel (A), as shown in Fig. 1.

After thermal equilibrium had been obtained about 1 hour later, tube B was drawn up to a constant level, and the stop cock (C) was reached above the surface of substrate solution. Ten minutes later, a piston rod (H) is pulled out, then the enzyme solution is poured into reaction vessel (A). Instantly they are mixed with each other and reaction starts. At the same moment, the temperature change in the reaction system is measured with time in order to obtain the reaction rates and the reaction heats.

On the other hand, reaction rates are also determined by mixing 10 ml of a diluted urease solution with each 140 ml of these two substrate solutions, which are prepared by dissolving the required amount of urea in the phosphate buffer of pH=6.7 and in a distilled water only. Each 0.5 ml of the mixture is pipetted out from the reaction solution after various periods of time, and the reaction in mixture is stopped by adding rapidly from 1 ml to 6 ml of 1/10 N-hydrochloric acid. The mixture is then treated by Conway's method, and the indophenol reagent method modified by Hatano[2], and the amount of ammonium nitrogen produced as result of the reaction is determined colorimetrically by using Hitachi photo-meter (Epo-B type) in various periods of time. By this treatment, the reaction rate, dx/dt, can be calculated.

The enzyme activity is examined as follows. After the stock enzyme solution dissolved at room temperature spontaneously is immersed into the thermostat adjusted to 40°±0.01°C, the enzyme activity in it is examined every 30 minutes by mixing the enzyme solution with the phosphate buffered 5% urea solution of pH=6.70 and measuring the time required to pH change 1.0 of the solution. From the test, it may safely be said that the enzyme activity of the stock solution is constant throughout the whole experiment.

It is also confirmed that in the case of the reaction of the enzymic solution deactivated by boiling it in the test tube, the temperature remains unchanged for 60 minutes in calorimetric measurement. This fact shows that, without the reaction by mixing the enzyme solution with the substrate solution, the temperature in the calorimeter remains constant for 60 minutes.

Results and Considerations

It can be shown that the temperature change with time is related by

$$\frac{dT}{dt} + K\Delta T = \frac{Q}{W} \cdot \frac{dx}{dt}, \qquad (1)$$

where dT/dt is the temperature change with time, dx/dt is the reaction velocity and K is a

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correction term for cooling. K in this apparatus is 0.0075. Q and W are the reaction heat and the water equivalent of the reaction system respectively. W is determined from the neutralization heat between NaOH and HCl experimentally.

The heat of reaction, Q, can be calculated by integrating equation (1) from $t=0$ and $t=t$, a certain time after the completion of the reaction,

$$Q=\frac{W}{a}\left\{\int_0^t dT+K\int_0^t \Delta T dt\right\}, \qquad (2)$$

where a is the initial concentration represented in mole unit.

The reactions in the presence of buffer and in the absence of buffer always proceed respectively, as shown shematically in Figs. 2, 3, 4, 5 and 6, and Figs. 7 and 8 respectively. From these results of the reactions in the two different solvents, the heats of reactions are calculated according to the equation (2), and they are tabulated in Table 2. The mean values of Q in urea concentration of 10^{-2} M/L and in reaction of $\sigma_{0.09}$-Urease are smaller than the others. It may be due to a difficulty of heat evolution for some of the lower concentration of urea and lower sensitivity of enzyme.

Consequently, it may be regarded as appropriate that the values of the reaction heat in the

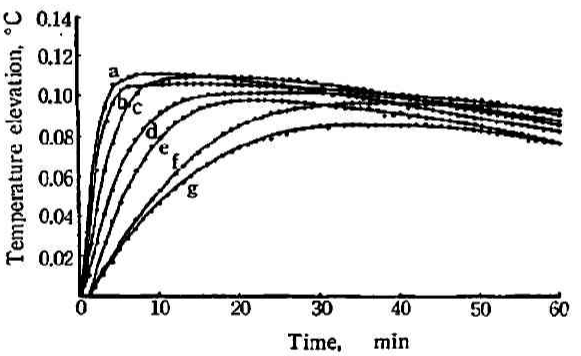
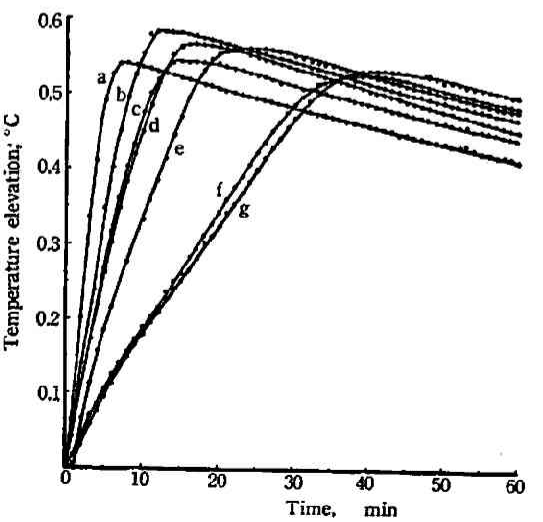


Fig. 2 Temperature and time relations for the reactions between various kinds of enzyme and urea of a concentration, 10^{-2} M/L in the presene of buffer pH = 6.70

a: enzyme U_6
b: 〃 U_{10}
c: 〃 $\frac{1}{2}U_6$
d: 〃 U_7
e: 〃 U_4
f: 〃 $\frac{1}{2}U_5$
g: 〃 $\sigma_{0.09}$

Fig. 3 Temperature and time relations for the reactions between various kinds of enzyme and urea of a concentration, 5×10^{-2} M/L in the presence of buffer pH = 6.70

a: enzyme U_6
b: 〃 U_8
c: 〃 $\frac{1}{2}U_6$
d: 〃 U_7
e: 〃 U_4
f: 〃 $\sigma_{00.4}$
g: 〃 $\frac{1}{2}U_5$



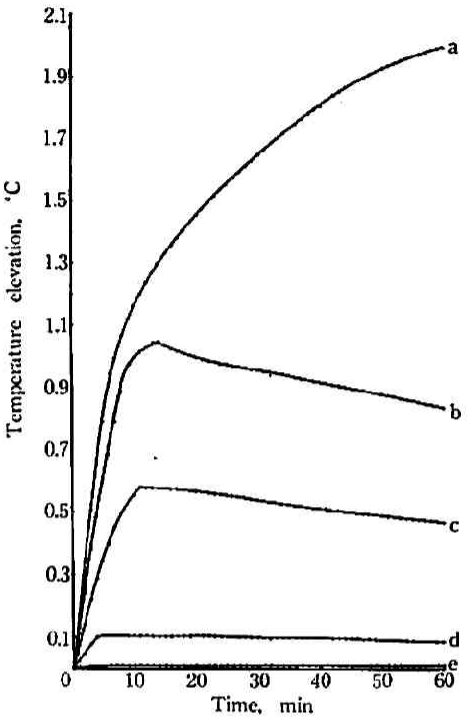


Fig. 4 Temperature and time relations for the reactions between enzyme U_8 and urea of various concentrations in the presence of buffer pH = 6.70

a: urea concentration 4×10^{-1} M/L
b: 〃 10^{-1} M/L
c: 〃 5×10^{-2} M/L
d: 〃 10^{-2} M/L
e: 〃 10^{-3} M/L

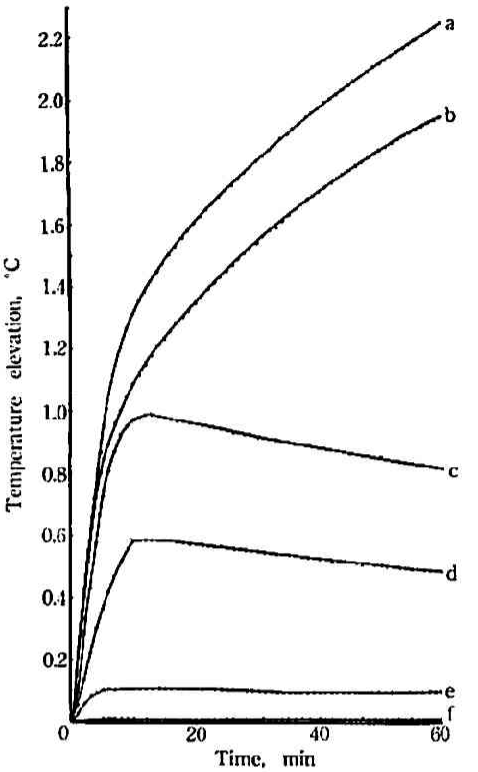


Fig. 5 Temperature and time relations for the reactions between enzyme U_{10} and urea of various concentrations in the presence of buffer pH = 6.70

a: urea concentration 1 M/L
b: 〃 4×10^{-1} M/L
c: 〃 10^{-1} M/L
d: 〃 5×10^{-2} M/L
e: 〃 10^{-2} M/L
f: 〃 10^{-3} M/L

Table 2 Heats of reactions. Q, kcal/M

Enzyme	Solvent	Initial concentration of urea M/L	Mean value of Q kcal/M
U_6, $\frac{1}{2}U_6$, U_7, U_8	Phosphate buffer	10^{-2}	13.670
U_4, $\frac{1}{2}U_5$, U_6, $\frac{1}{2}U_6$, U_7, U_8	〃	5×10^{-2}	14.426
U_8	〃	10^{-3}, 10^{-2}, 5×10^{-2}, 10^{-1}	14.085
U_{10}	〃	10^{-3}, 10^{-2}, 5×10^{-2}, 10^{-1}	14.289
$\sigma_{0.09}$	〃	〃	13.865
U_9	None buffer	〃	4.258
$\sigma_{0.09}$	〃	〃	3.866

concentration of 5×10^{-2}M/L and in the case of U_8, U_{10}-Urease are prefered.

The mean value thus obtained is 14.266kcal/M. The value of Q in the reaction of non-buffered solution, however, is 4.258kcal/M, as shown in Table 2. Consequently the difference

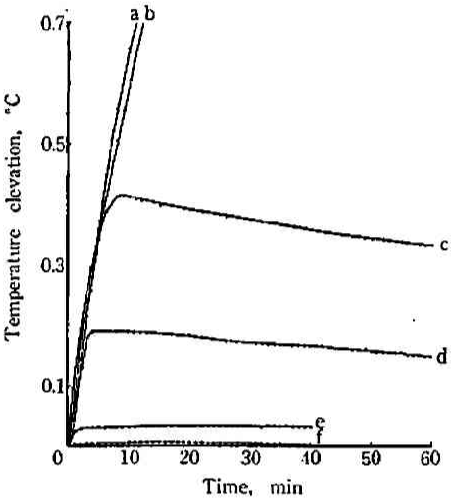


Fig. 6 Temperature and time relations for the reactions between enzyme U_9 and urea of various concentrations in the absence of buffer

a: urea concentration 1 M/L
b: 〃 4×10^{-1} M/L
c: 〃 10^{-1} M/L
d: 〃 5×10^{-2} M/L
e: 〃 10^{-2} M/L
f: 〃 10^{-3} M/L

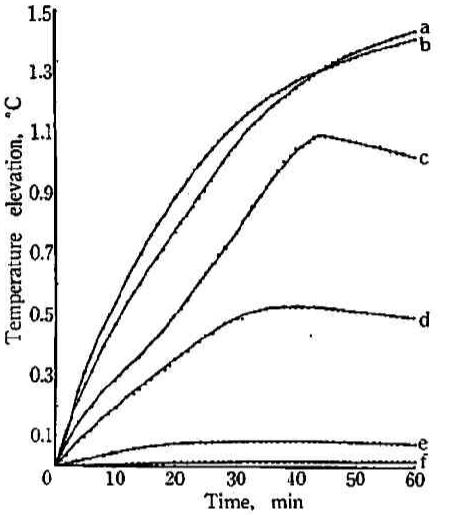


Fig. 7 Temperature and time relations for the reactions between enzyme $\sigma_{0.08}$ and urea of various concentrations in the presence of buffer pH=6.70

a: urea concentration 4×10^{-1} M/L
b: 〃 1 M/L
c: 〃 10^{-1} M/L
d: 〃 5×10^{-2} M/L
e: 〃 10^{-2} M/L
f: 〃 10^{-3} M/L

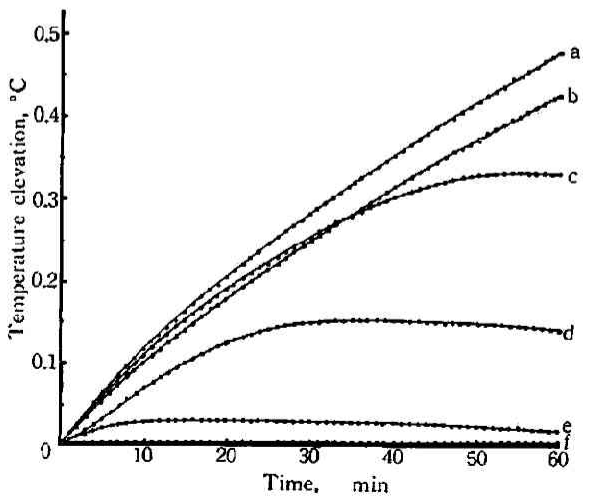


Fig. 8 Temperature and time relations for the reactions between enzyme $\sigma_{0.09}$ and urea of various concentrations in the absence of buffer

a: urea concentration 1 M/L
b: 〃 4×10^{-1} M/L
c: 〃 10^{-1} M/L
d: 〃 5×10^{-2} M/L
e: 〃 10^{-2} M/L
f: 〃 10^{-3} M/L

of reaction heats in these two solvents is 10.008 kcal/M.

From the analysis of NH_3, it is found that an amount of NH_3, produced as result of the reaction in the absence of buffer, *i. e.* in aqueous urea solution, is practically equal to that value in the presence of buffer. It shows that the extent of the two reactions is almost equal in these two solvents.

Hence it strikes me that the above difference of Q must originate from the result of reaction between NH_3 produced as result of the reaction and the phosphate buffer presented previously in the reaction solution. Then the reactions between a known concentration of NH_3 and a phosphate buffer in the absence of urea were investigated, and the results as shown in Fig. 9 were obtained. The heat produced as result of this neutralization is tabulated in Table 3. The heat of the

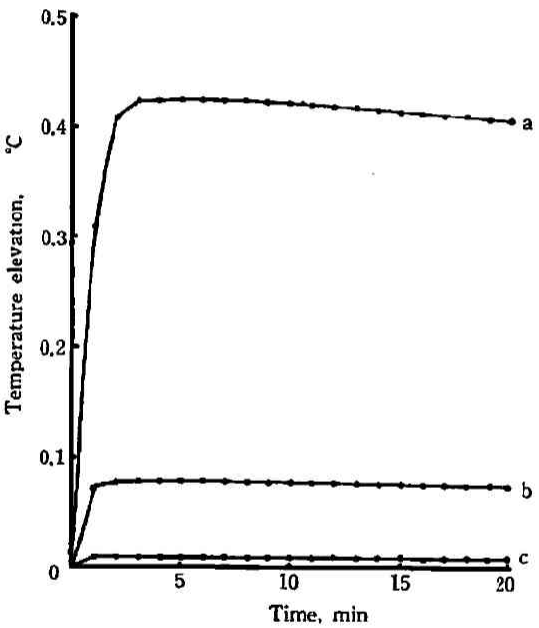


Fig. 9 Reaction curve between NH_3 and the buffered solution of pH = 6.70

a : ammonia concentration 5×10^{-2} M/L
b : 〃 10^{-2} M/L
c : 〃 10^{-3} M/L

Table 3 Heat of neutralization between NH_3 and the phosphate buffer solutions

Concentration of NH_3 M/L		Heat of neutralization kcal/M
5×10^{-2}		10.293
10^{-2}		9.308
10^{-3}		10.950
	mean	10.187

neutralization calculated from the above data concerning the reaction between NH_3 and buffer was 10.187 kcal in the mean. Namely it is found from this result that the difference of reaction heats in the two different solvents, *i. e.*, in the presence of buffer and in the absence of buffer, corresponds to the reaction heat between NH_3 and the phosphate buffer solution. From this fact, the reaction heat

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of the urea-urease system is evidently equivalent to the reaction heat in the absence of buffer.

On the other hand, the heat of this reaction can be calculated from the value of chemical table theoretically as follows. Combustion reaction of urea follows the stoichiometric equation,

$$CO(NH_2)_2(s)+3/2O_2(g)=CO_2(g)+2H_2O(l)+N_2(g)+151.7\,kcal\,.$$

The heat of formation of $CO(NH_2)_2(s)$ can be calculated from the above equation by assuming that the heats of formation of $CO_2(g)$ and $H_2O(l)$ are -94.4 kcal/M and -68.4 kcal/M respectively. That is

$$\Delta H=-79.1\,kcal/M\,.$$

And the reaction heat between $CO(NH_2)_2(s)$ and water can be also calculated from the difference of the heat of formation after and before the reaction systems as the following chemical formula,

$$CO(NH_2)_2(s)+H_2O(l)=CO_2(g)+2NH_3(g)\,,\qquad \Delta H=31.5\,kcal/M\,,$$

namely $$CO(NH_2)_2(s)+H_2O(l)=CO_2+2NH_3(g)-31.5\,kcal\,.$$

however $$CO(NH_2)_2(s)+aq=CO(NH_2)_2(aq)-3.69\,kcal\,.$$

therefore $$CO(NH_2)_2(aq)+H_2O(l)=CO_2(g)+2NH_3(g)+aq-27.81\,kcal\,,$$

$$\Delta H=27.81\,kcal/M\,.$$

It is an endothermic reaction of 27.81 kcal/M, while NH_3 and CO_2 dissolve into the solution and generate the heats of dissolution. That is,

$$2NH_3(g)+aq=2NH_3(aq)+2\times8.35\,kcal\,,$$

$$CO_2(g)+1700H_2O=CO_2(aq)+5.88\,kcal\,.$$

And one mole of $2NH_3(aq)$ neutralizes $CO_2(aq)$ produced in the same time, and generates the heat of neutralization.

$$NH_3(aq)+CO_2(aq)=(NH_4)HCO_3(aq)+H_2O+9.73\,kcal\,.$$

Consequently the aggregate of these reaction heats is the amount of the heats evolved during this reaction. It is 4.50 kcal/M in total.

If one examines the ionization constant for carbonic acid, one finds that the second, $K_2=4.7\times10^{-11}$ is much smaller than the first, $K_1=4.31\times10^{-7}$, *i. e.*, about one-ten thousandth difference. This means that for practical purposes the contribution to the oxonium ion concentration from the second ionization may be neglected.

Consequently the residual ammonia after the neutralization is completed, gives rise to the increase of the pH value of the reaction solution. It is remarkable in an aqueous solution, *i. e.*, in the absence of buffer, as shown in Fig. 10. These curves are the plots of pH values which are changed with time by NH_3 produced as result of urea and urease reaction in the absence of buffer (these are measured by a Hitachi pH meter; EHP-I tpye).

Now the theoretical value of the reaction heat calculated from the above equations, 4.50

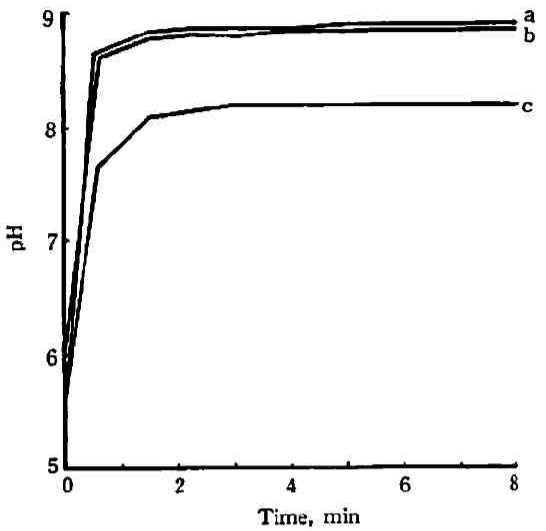

Fig. 10 Plots of pH value of reaction solution *vs.* time

a : enzyme or $\sigma_{0.09}$-urea concent. 5×10^{-2} M/L
b : 〃－〃 10^{-2} M/L
c : 〃－〃 10^{-3} M/L

kcal/M, is in good agreement with the value of the reaction heat 4.258kcal/M, calculated from dT/dt curve in the reaction of aqueous solution as previously estimated.

A number of hypotheses about urease action have been reported. Yamasaki[5] showed that carbamate as an intermediate product is formed when urease decomposes urea.

Mack and Villers[6] found cyanic acid to be produced in this reaction, but concluded this to be the result of a side reaction and decided that the intermediate product is ammonium carbamate. Fearon[7] advanced the theory that urease decomposes urea to an ammonium cyanate, and the cyanate then is assumed to be hydrolyzed spontaneously to form NH_3 and CO_2. However Sumner objected to the hypothesis. Previously Kay[8] had objected to Fearon's hypothesis.

Sumner[9] *et al* demonstrated conclusively that when urea is decomposed by crystalline urease, ammonium carbamate is formed in the absence of buffer, and then decomposes, but if neutral phosphate is present no carbamate can be detected. Namely the first products which are produced when urea is decomposed by urease are CO_2 and NH_3, and these substances unite in the absence of buffer to form ammonium carbamate, while in the presence of buffer no carbamate results. In other words he concluded that ammonium carbamate is an intermediate product, but it is not necessarily the first intermediate product. Bersin, Köster and Brandt[10] assumed an intermediate addition compound in the first step of the reaction, which decomposes into carbamic acid and urease in the second step. However the product in the first step is indeed a pressing question in this case.

5) E. Yamasaki, *Sci. Reps. Tohoku Imperial Univ., Ser.* 1., 9, 96 (1920)
6) E. K. Mack and D. S. Villers, *J. Am. Chem. Soc.*, 45, 505 (1923)
7) W. R. Fearon, *Biochem. J.*, 30, 1652 (1936); 17, 84, 800 (1923)
8) H. D. Kay, *ibid.*, 17, 277 (1923)
9) J. B. Sumner, D. B. Hand and R. G. Holloway, *J. Biochem.*, 91, 333 (1931)
10) T. Bersin, *Kurzes Lehrbuch der Enzymologie*, 2nd ed., p. 69, Akadem. Verlagsgesellshaft, Leipzig, (1937)

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The behaviour towards urease action is almost the same in the absence of buffer as in the presence of buffer in the present experiments, and the value of reaction heat calculated from the chemical formula by assuming theoretically that NH_3 and CO_2 are the first products of the reaction agrees very closely with the heat evolved in the absence of buffer.

If special regard is also paid to the fact that carbamic acid and carbamate are very unstable substances in water, it may be concluded that urea molecule absorbed on urease surface loses simultaneously the resonance[11] of the molecule, and instantly decomposes very readily into CO_2 and NH_3, and then these two gases are dissolved into the solution, and neutralized each other to produce $(NH_4)HCO_3$ as a final product.

Next object is the determination of reliability of the Michaelis-Menten law, by investigating the reaction rate from the experiments of thermal analysis. From the results of the two different measurements as previously stated, it is ascertained that the temperature change with time, dT/dt, is in good proportionality with the rate, dx/dt, obtained from the determination of NH_3 produced as result of reaction by the Conway's and the indophenol reagent methods modified by Hatano[2].

Fig. 11 shows the results of such measurements. The signs of a and b in the figure show

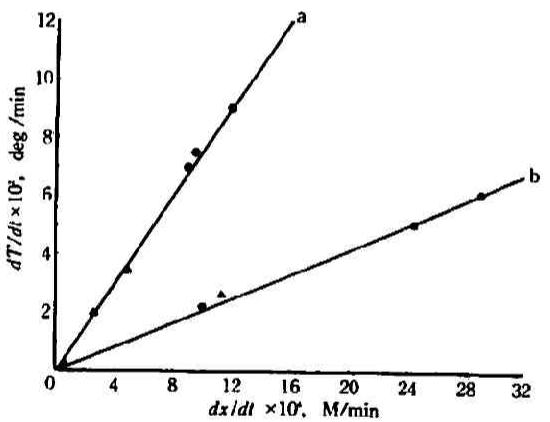

Fig. 11 Temperature change with time, dT/dt *vs.* reaction rate, dx/dt

a { ⊙: Enzyme U_{10}—phosphate buffer pH = 6.70—urea concentration 5×10^{-2} M/L
△: 〃—urea concentration 10^{-1} M/L

b { ⊙: Enzyme U_9—none buffer—urea concentration 5×10^{-2} M/L
△: 〃—〃—urer concentration 10^{-2} M/L

the relations for reaction in presence of the phosphate buffer and reaction in the absence of buffer respectively. It makes a straight line passing the origin, and the reaction heat, Q, can be calculated from the inclination of the line obtained by using equation (1). Reaction heats in the presence of buffer and in the absence of buffer are 14.308 and 3.738 kcal/M respectively. These values are almost in agreement with those calculated from the integration of dT/dt curve in the calorimetric measurements.

The present author will discuss the rate of this reaction with the temperature change, dT/dt, instead of the decomposition rate of urea, dx/dt.

The initial rate is in good proportionality with the enzyme activity and concentration, as shown in Fig. 12. The signs of a and b in the figure show the relation of the initial rate *vs.* enzyme activity in the urea concentration of 5×10^{-2} M/L and 10^{-1} M/L respectively.

11) H. B. Bull, *Physical Biochemistry*,, 2nd Ed., p. 15, John Wiley & Sons, Inc., New York (1951)

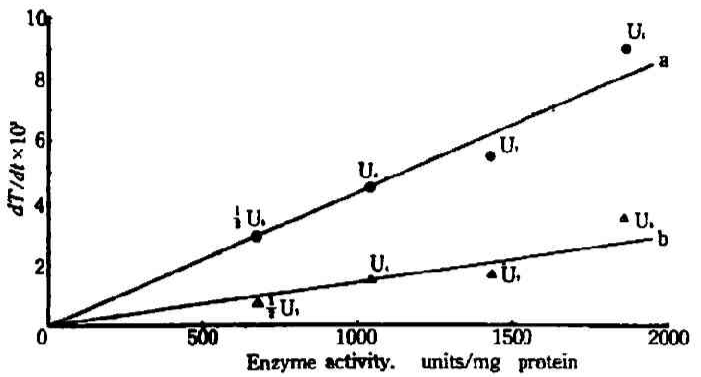

Fig. 12 Plots of initial rate *vs.* enzyme activity in the urea concentrations
a: 5×10^{-2} M/L
b: 10^{-2} M/L

Initial rates are measured in the phosphate buffer of pH=6.70 at 40°±0.001°C over the range of urea concentrations from 10^{-3} to 1 M/L. Initial rate preferred the rate at a minute after the reaction started to the one at the start immediately. The lower rate at the start seems to be due to the time lag of Beckmann's thermometer. The plots of the initial rates versus urea concentrations are found to fulfil a curve of the characteristic reaction form obeying the Michaelis-Menten law up to 4×10^{-1} M/L, after which there is a falling-off of the rate as shown in Fig. 13.

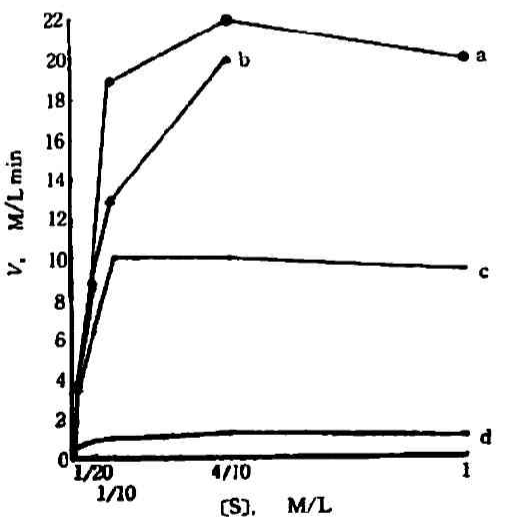

Fig. 13 Plots of initial rate *vs.* concentration of urea at 40°±0.001°C
a: Enzyme U_{10}—phosphate buffer of pH=6.70
b: 〃 U_5— 〃
c: 〃 U_0—none buffer
d: 〃 $\sigma_{0.09}$—phosphate buffer of pH=6.70

These plots are also investigated in the case of the reaction in the absence of buffer. In this case, a falling-off of the rate begins at the urea concentration of 10^{-1} M/L. The same relation was found by Laidler[12] and Wall[13] who investigated the reaction in a phosphate buffer and in an inert buffer, *i.e.*, THMAM-H_2SO_4 buffer. Laidler explained this falling-off of the rate at high urea concentration in terms of model in which a urea molecule and a water molecule must become adsorbed on the neighboring sites on the surface of urease molecule, and the rate should increase

12) K. J. Laidler and J. P. Hoare, *J. Am. Chem. Soc.*, 71, 2699 (1949)
13) M. C. Wall and K. J. Laidler, *Arch. Biochem. Biophys.*, 43, 299 (1953)

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with urea concentration up to a certain point, but at high concentration the urea becomes adsorbed on both sites preferentially, and therefore the reaction is inhibited. He derived the rate equation from his hypothesis obeying the Langmuri adsorption isotherm.

On the other hand, Henri[14], Brown[15], and Van Slyke[16] suggested previously that at high concentration of substrate the rate determining step relates to the enzymic reaction itself, but not to concentrations. But it may be considered that in the present experiment the retarding effect of NH_3 produced by the reaction occurs at a very high urea concentration, even in initial rate. The author made some studies of ammonium ion effect and found that in the reactions between urease U_{10} and urea ammonium solutions namely 5×10^{-2} M/L, and 10^{-1}M/L which are prepared by dissolving each urea in an ammonium water of concentrations of 10^{-1}M/L and 2×10^{-1}M/L respectively, the initial rate decreased to about one third of the case of non-ammonium solution. On making a comparison, it is found that in the maximum points of initial rate, non-buffered solution lies in lower concentration of urea than buffered solution, as shown in Fig. 13. The final pH of the reaction solution in the non-buffered case passes over pH=9 in far lower urea concentration than the final one in the buffered case, as shown in Table 4.

Table 4 Final pH of the reaction solution

Initial concentration of urea M/L	Final pH of the reaction solution			
	Non-buffered		Buffered	
	U_9	$\sigma_{0\text{-}09}$	U_{10}	$\sigma_{0\text{-}09}$
10^{-3}	7.27	8.32	6.92	6.73
10^{-2}	9.80	9.20	7.22	6.87
5×10^{-2}	9.93	9.45	7.40	7.25
10^{-1}	9.94	9.43	9.30	8 50
4×10^{-1}	9.92	9.56	9.93	9.35
1	9.91	9.81	10.00	9.96

The reaction type in the substrate concentration over 4×10^{-1}M/L is considerably different from one below this concentration. This difference is partly due to the evaporation of NH_3 from the reaction solution. Laidler[12,17] measured initial rate at a variety of ammonium ion concentrations in order to determine the nature of the inhibition and found that the reaction was inhibited by ammonium ion. From these results, the author concluded that the falling-off of the rate was due to the retarding effect of NH_3 at a very high urea concentration.

The plots of the reciprocals of the initial rates versus the reciprocals of substrate concentrations are found to give a curve of linear form from 10^{-2} up to 4×10^{-1}M/L of substrate concentration, but at 1M/L to give a horizontal curve as shown in Fig. 14.

The Michaelis constants in the presence of buffer and in the absence of buffer are determined from these linear relationships by using the Dixon's method[3]. These values are 6.3×10^{-2}M/L and 3.2×10^{-2}M/L respectively, and the values of other kinds of urease are almost equal to the above

14) V. Henri, *Lois générales de l'action des diastases*, Paris (1903)
15) A. J. Brown, *Trans. Chem. Soc.*, **81**, 373 (1902)
16) D. D. Van Slyke, *Advances in Enzymology*, **2**, 33 (1942)
17) K. J. Laidler and J. P. Hoare, *J. Am. Chem. Soc.*, **72**, 2487 (1959)

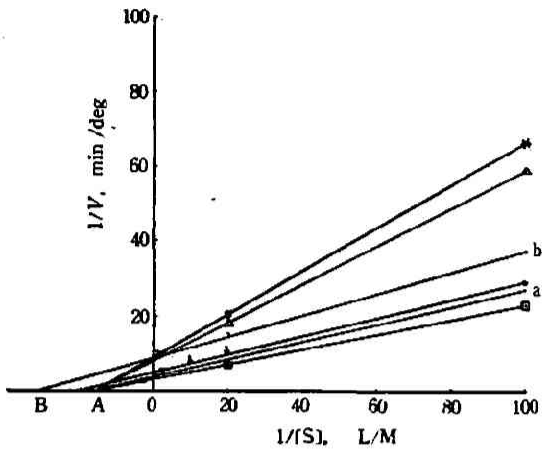

Fig. 14 Plots of reciprocals of initial concentration *vs.* substrate concentration
$B=3.2\times10^{-2}$ $A=6.3\times10^{-2}$
* U_4—buffer—pH=6.70
△ U_7—buffer—pH=6.70
● U_8—buffer—pH=6.70
⊡ U_6—buffer—pH=7.00

data, as shown in Fig. 14. Moelwyn-Hughes[18] reported previously the value at 25°C and it was 2.5×10^{-2}. The reciprocal values obtained by Laidler[13] in the THMAM–H_2SO_4 buffer were 250 at pH=7.13 and 256 at pH=8.00 respectively.

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18) E. A. Moelwyn-Hughes, *Hdb. Enzymolg.*, p. 260 (1940)