into ice and water, yielding 1.5 g. (58%) il of m. p. 265–280°. Recrystallization gave golden yellow crystals of m. p. 285°. e product with phenylhydrazine in eth- crystals of m. p. 229–230°.[8]

Summary

benzyl chloride reacts with benzene : of aluminum chloride to give ben- benzene, with potassium acetate in give pentachlorobenzyl acetate, and ydroxide or sodium alkoxides in al- ethers. With sodium hydroxide in aqueous acetone, dipentachlorobenzyl ether and small amounts of 4-pentachlorophenylbutanone-2 were obtained.

Pentachlorobenzal chloride undergoes the Friedel–Crafts alkylation to give benzhydrylpentachlorobenzene. It does not react with potassium acetate or silver acetate in acetic acid or with potassium hydroxide in aqueous acetone. With sodium alkoxides in alcohols acetals of 2,3,5,6-tetrachloro-4-alkoxybenzaldehydes are obtained. These on hydrolysis give the corresponding aldehydes.

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TRIBUTION FROM CHEMISTRY DEPARTMENT, NORTHWESTERN UNIVERSITY MEDICAL SCHOOL]

Peptic Hydrolysis of Egg Albumin. I. Kinetic Studies

BY HENRY B. BULL AND BYRON T. CURRIE

been the subject of exhaustive does not appear, however, that the hydrolysis of a purified protein by n studied in detail. We have inves- ion of crystalline pepsin on crystal- lin as a function of substrate con- hydrogen ion concentration and of An interpretation of these data is d the Michaelis–Menten[2] formula- extended to include the influence of and the formation of an activated

Experimental

l to Armour and Company for the crys- Its activity as determined by us was 0.19 of nitrogen. The egg albumin was pre- hen's eggs by the method of Kekwick and

the action of pepsin on egg albumin was he amount of egg albumin which cannot ed at its isoelectric point. The technique The egg albumin solution was brought to by the cautious addition of hydrochloric ion was placed in the reaction vessel and perature in a thermostated bath. The of solution containing sufficient pepsin pepsin per 100 cc. of reaction mixture slowly stirred. A glass electrode was in- reaction mixture and the *p*H maintained bbling hydrochloric acid gas through the . A small amount of caprylic alcohol vent foaming. Ten-cc. samples were re- als to 50-cc. Pyrex volumetric flasks and ately to the isoelectric point of egg albu- lition of acetate buffer. These samples a glycerol bath to boiling, cooled and ne, filtered, and the total nitrogen of an iltrate determined with micro-Kjeldahl. as expressed in terms of protein and is lubilized protein.

Results

cal values are so extensive that it is not practical to report them in detail, and we are characterizing our data by means of the Michaelis–Menten constants. These constants were obtained by plotting the reciprocal of the initial rate of reaction against the reciprocal of the egg albumin concentration. The intercept of the straight lines on the *y*-axis gave the reciprocal of the maximum velocity (V), and the slopes of the straight lines yielded the ratio of the dissociation constant (K_m) of the Michaelis–Menten complex to the maximum velocity. The initial reaction rates were obtained from the slope of the line at zero time when the amount of solubilized protein was plotted against time. At 30° these plots gave essentially straight lines up to one hour, while at 45° the extent of reaction was so much greater that gentle curves were obtained. Three to four determinations for each protein concentration at various time intervals were needed to evaluate the initial rate, and four to five protein concentra-

TABLE I

MOLE-SECOND MICHAELIS–MENTEN CONSTANTS FOR THE DIGESTION OF EGG ALBUMIN BY PEPSIN AT 30°

*p*H	*V*	K_m
1.34	0.092	0.67×10^{-4}
1.60	.092	1.78×10^{-4}
1.80	.074	2.44×10^{-4}
2.00	.077	3.40×10^{-4}
2.40	.038	4.45×10^{-4}
2.60	.030	5.95×10^{-4}
2.80	.013	7.35×10^{-4}

TABLE II

MOLE-SECOND MICHAELIS–MENTEN CONSTANTS FOR THE DIGESTION OF EGG ALBUMIN BY PEPSIN AT 45°

*p*H	*V*	K_m
2.00	0.48	3.94×10^{-4}
2.30	.37	4.45×10^{-4}
2.63	.16	5.07×10^{-4}
2.85	.09	5.82×10^{-4}

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tions extending from 0.5 to 6.0% were used to estimate the Michaelis–Menten constants. Thus, the evaluation of each constant involved 12 to 20 separate analyses. The maximum velocity (V) was expressed in moles of egg albumin solubilized per second per mole of pepsin. The molecular weight of egg albumin was taken as 45,000 and that of pepsin as 35,000. The dissociation constant (K_m) was expressed in molar concentrations. These values are shown in Table I for 30° and in Table II for 45°.

Discussion

According to the theory of absolute reaction rates, the rate of decomposition of the activated complex should be

$$AC^*(kT/h)$$

where A is a coefficient whose value approaches unity, C^* is the concentration of the activated complex, k is Boltzmann's constant, h is Planck's constant and T is the absolute temperature. The important question arises as to whether or not the Michaelis–Menten complex is identical with the activated complex. If this be true, then V should equal $AkT/K_m h$. Setting A equal to unity and substituting the value of K_m at pH 2.0 and at 30° (see Table I) and the values for the constants, we calculate that V should be 1.86×10^{17} moles per second per mole of pepsin. Evidently, the Michaelis–Menten complex is not the activated complex; its concentration is very much greater than that of the activated complex. It is, therefore, necessary that the Michaelis–Menten complex pass over into an activated complex before the egg albumin can hydrolyze into peptides.

The Michaelis–Menten treatment also neglects the influence of the hydrogen ions. Since the reaction rate is so dependent on the concentration of hydrogen ions, it seems reasonable to assume that they enter directly into the reaction.

There are, no doubt, a variety of ways to formulate the above considerations; three simple possibilities are

(1) $H^+ + E \underset{K_1}{\rightleftharpoons} H^+E$;

$H^+E + S \underset{K_2}{\rightleftharpoons} H^+ES \underset{K_3}{\rightleftharpoons} [H^+ES]^* \longrightarrow E + P$

(2) $H^+ + S \underset{K_1}{\rightleftharpoons} H^+S$;

$H^+S + E \underset{K_2}{\rightleftharpoons} H^+ES \underset{K_3}{\rightleftharpoons} [H^+ES]^* \longrightarrow E + P$

(3) $E + S \underset{K_1}{\rightleftharpoons} ES$;

$ES + H^+ \underset{K_2}{\rightleftharpoons} H^+ES \underset{K_3}{\rightleftharpoons} [H^+ES]^* \longrightarrow E + P$

In the above, E represents the enzyme, S the egg albumin, H^+ the hydrogen ion and $[H^+ES]^*$ is the activated complex.

A consideration of the first and second formulation reveals that the maximum velocity of the reaction should be independent of the hydrogen ion concentration. This is contrary to experience, so that both of these formulations must be rejected.

The third formulation will be considered in detail. The velocity of the reaction should be

$$v = \frac{AkT}{h}[H^+ES]^* \qquad (1)$$

Letting K_1 equal $E \times S/ES$, K_2 equal $H^+ \times ES/H^+ES$, K_3 equal $H^+ES/[H^+ES]^*$ and K equal $K_1 \times K_2 \times K_3$, we have, after substituting the values of the dissociation constants in equation 1 and rearranging

$$\frac{1}{v} = \frac{Kh}{AkTHSE_0} + \frac{Kh}{AkTK_1HE_0} + \frac{Kh}{AkTK_1K_2E_0} + \frac{h}{AkTE_0} \qquad (2)$$

where E_0 is the total molar enzyme concentration. Evidently, when E_0 is unity and $1/v$ is plotted against $1/S$, $Kh/AkTH$ is equal to the slope of the line and is equal to K_m/V. The intercept on the y-axis is

$$\frac{1}{V} = \frac{Kh}{AkTK_1H} + \frac{Kh}{AkTK_1K_2} + \frac{h}{AkT} \qquad (3)$$

When $1/V$ is plotted against $1/H^+$, the slope of the line is $Kh/AkTK_1$, and the intercept is $Kh/AkTK_1K_2 + h/kTA$. It is thus possible to evaluate all the dissociation constants of the third formulation, and these are given in Table III for 30° and in Table IV for 45°. The value of K_3 is based upon the assumption that A is unity. Since we have the value of the dissociation constants at two different temperatures, it is possible to calculate the heats of dissociation as well as the entropy changes involved.

TABLE III

DISSOCIATION CONSTANTS AND ENERGIES FOR STEPS INVOLVED IN THE DIGESTION OF EGG ALBUMIN BY PEPSIN AT 30°

Constant	ΔF_{25} Calories	ΔH Calories	ΔS E. U.
$K_1 = E \times S/ES =$ 7.43×10^{-4}	4,350	− 1,450	−19.2
$K_2 = H^+ \times ES/H^+ES =$ 7.17×10^{-3}	2,980	11,100	26.6
$K_3 = H^+ES/[H^+ES]^* =$ 5.7×10^{13}	−19,150	−31,400	−40.5

TABLE IV

DISSOCIATION CONSTANTS FOR STEPS INVOLVED IN THE DIGESTION OF EGG ALBUMIN BY PEPSIN AT 45°

$K_1 = E \times S/ES = 6.63 \times 10^{-4}$
$K_2 = H^+ \times ES/H^+ES = 1.71 \times 10^{-2}$
$K_3 = H^+ES/[H^+ES]^* = 4.89 \times 10^{12}$

The heat of dissociation of the proton from the enzyme-substrate complex is about 11,000 calories and its pK_a is about 2.14. The dissociation constant is about what is to be expected from the ionization of a carboxyl group, but the heat change involved is much too large. It can be seen that the plot of the maximum velocity against pH is essentially a titration curve of the complex.

Figure 1 shows a plot of the maximum velocities against *p*H at 30°. Also shown are the maximum velocities calculated according to equation 3. The agreement between the experimental and theoretical values is satisfactory.

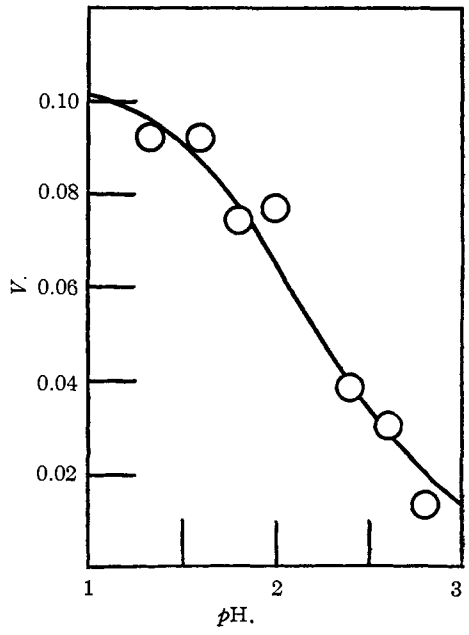

Fig. 1.—Plot of experimental points for the maximum velocity of digestion of egg albumin against *p*H for 30° (see Table I). The solid line has been calculated from equation 3.

The large heat needed for the creation of the activated complex is very nearly equal to the heat of activation for the denaturation of egg albumin which, at 30° and in the acid region, is about 35,000 calories.[4] The creation of the activated complex quite possibly involves changes in the entire egg albumin molecule and probably is not confined to a single peptide bond.

The influence of denaturation of the egg albumin on our results has been of concern to us. It should be pointed out that the rates of hydrolysis reported are initial rates obtained by extrapolating the time rate curves to zero time. The influence of denaturation should, therefore, be minimal.

As we have formulated the hydrolysis of egg albumin by pepsin, the collision rate between the pepsin and the egg albumin molecules becomes secondary. It appears, however, that the Smoluchowski[5] theory of the precipitation of colloida solutions provides a more realistic approach to the calculation of the collision rate than does the gas collision formula.

According to Smoluchowski, the number of unit particles in a colloid at any time, t, is given by

$$n = n_0/(1 + 8\pi r D n_0 t) \qquad (4)$$

where r is the radius of the unit particle, D is the diffusion constant, n_0 is the number of unit particles at the beginning of the reaction. Evidently, the rate of disappearance of unit particles is twice as great as the collision rate and is at zero time equal to $-8\pi r D(n_0)^2$. Then the collision rate is $4\pi r D(n_0)$.[2] Calculations show that the rate of collision of pepsin molecules with egg albumin molecules under the condition which we have worked is about $^1/_{200}$th of the rate as given by the gas collision theory.

We have investigated the effect of adding to the reaction mixture a concentrated filtrate of solubilized protein. At *p*H 2.0 and in the presence of one mg. of pepsin per 100 cc., 1.70 g. of solubilized protein gave a maximum velocity of 0.036 mole per second per mole of pepsin, and the K_m was 2.82×10^{-5}. These are to be contrasted with the corresponding values shown in Table I obtained in the absence of solubilized protein. Evidently, the soluble peptides are non-competitive inhibitors of the reaction.

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Summary

1. The rate of digestion of egg albumin by pepsin has been investigated as a function of substrate concentration, of *p*H and of temperature.

2. The amount of protein which cannot be heat-coagulated at the isoelectric point of egg albumin is taken as a measure of the extent of digestion.

3. It is concluded that the activated complex is not identical with the Michaelis–Menten complex. It is also concluded that the hydrogen ions enter directly into the catalytic reaction, and the reaction mechanism has been formulated accordingly. The Michaelis–Menten treatment of enzyme reactions is thus extended.

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(4) Cubin, *Biochem. J.*, **23**, 25 (1929).

(5) Smoluchowski, *Physik. Z.*, **17**, 557, 583 (1916).