power, iodine absorption and other properties is in all probability the enolic form of the semialdehyde of malonic acid. The crystalline acid can be partially sublimed without decomposition but undergoes upon heating a considerable degree of polymerization. It yields formic and oxalic acids upon oxidation and is exceedingly unstable in the presence of organic impurities.

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THE RATE OF HYDROLYSIS OF CASEIN IN ACID SOLUTIONS AS MEASURED BY THE FORMATION OF AMINO NITROGEN

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RECEIVED OCTOBER 3, 1928 PUBLISHED MARCH 6, 1929

Introduction

Greenberg and Burk[1] from their own measurements and such data as were available, calculated the order of the reaction of the hydrolysis of the proteins in solutions of acids. The hydrolysis for gelatin, silk fibroin and gliadin was found to conform to the equation of a second order reaction. A single series of measurements on casein by Dunn[2] apparently fitted a first order reaction best. This was in agreement with the findings of Carpenter,[3] who worked, however, under radically different conditions. It seemed worth while to check the order of the reaction of the hydrolysis of casein, since if a universal structural basis is common to all proteins, individual proteins would not be expected to show different orders of hydrolysis under the same fixed conditions. From the results given in the following, it is to be seen that casein conforms to the proteins already measured and that the hydrolysis reaction is of the second order under the conditions employed. Also, as was found for the other proteins, the effect of acids in accelerating the rate of hydrolysis is shown to be proportional to the hydrogen-ion activity of the acid solutions.

Experimental

The measurements were carried out at the temperatures of 127.5, 117.5 and 105.5°, employing the acids, hydrochloric, sulfuric and phosphoric. The hydrolyses were carried out in an autoclave with a steam line the pressure of which could be regulated by a reducing valve. The casein used was prepared according to the method, somewhat modified, of Van Slyke and Baker.[4] One-gram samples of thoroughly dried casein were placed in 50-cc. pyrex Erlenmeyer flasks, 40 cc. of the desired acid was added and

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[1] D. M. Greenberg and N. F. Burk, THIS JOURNAL, **49**, 275 (1927).

[2] M. S. Dunn, *ibid.*, **47**, 2564 (1925).

[3] D. C. Carpenter, *J. Biol. Chem.*, **67**, 647 (1926).

[4] L. L. Van Slyke and J. C. Baker, *ibid.*, **35**, 128 (1918); D. M. Greenberg and C. L. A. Schmidt, *J. Gen. Physiol.*, **7**, 287 (1924).

then the flasks were sealed with a blast lamp. This made the casein concentration in the acids 2.5%. In a few instances large pyrex test-tubes were employed, using half the above amounts of casein and acid. A series of such sealed flasks or tubes was placed in the autoclave and the steam turned on. At appropriate intervals duplicate flasks were removed from the autoclave and immediately immersed in ice water. The removal of these flasks was accomplished in about eighty seconds, so that the temperature of the remaining flasks did not fall appreciably before the temperature was restored to the working level. As soon as the flasks were cool, the seal was broken and the contents neutralized to prevent further hydrolysis. With the gram samples the final volume was made up to 100 cc., with the half-gram samples up to 50 cc. One-cc. amounts of these solutions were used for analysis of amino nitrogen by the Van Slyke method.[5] Duplicate analyses were made on each sample. As in the previous work, the increase in amino nitrogen was taken as measuring the amount of hydrolysis. In calculating the percentage of hydrolysis, the figure taken for total amino nitrogen was that given by Dunn, namely, 70% of the total nitrogen of casein.

The experimental results are given in Tables I to V. The column headings in the tables require no further explanation.

TABLE I

THE HYDROLYSIS OF CASEIN BY HYDROGEN CHLORIDE AT 127.5°

1.0 *N* Hydrochloric Acid

Time, hours	0.5	0.75	1.0	1.25	1.5	1.75	2.0
Amino N per g. of casein, mg.	43.3	51.2	56.3	61.4	65.2	68.2	68.9
Hydrolysis, %	45.7	54.3	59.5	64.8	68.9	72.2	72.8
K, vel. const., 1st order	0.53	0.46	0.39	0.36	0.34	0.32	0.28
K, vel. const., 2d order × 10^3	17	16	15	15	15	15	13
K, vel. const., 3d order × 10^4	2.4	2.5	2.6	2.8	3.1	3.4	3.2

$k/a_{\pm} = 0.019$. Average of second order constants = 15×10^{-3}.

0.5 *N* Hydrochloric Acid

Time, hours	0.5	0.75	1.0	1.25	1.5
Amino N per g. of casein, mg.	31.3	37.5	44.4	46.9	50.1
Hydrolysis, %	33.1	39.6	46.9	49.6	52.8
K, 2d order const. × 10^3	9.9[a]	8.7	8.8	7.9	7.5

[a] Value not used in obtaining average.

$k/a_{\pm} = 0.021$. Average of constants, 8.2×10^{-3}.

TABLE II

THE HYDROLYSIS OF CASEIN BY HYDROGEN SULFATE AT 127.5°

2.5 *N* Sulfuric Acid

Time, hours	0.5	0.75	1.0	1.25	1.5	1.75	2.0	6.5
Amino N per g. of casein, mg.	47.2	54.5	58.8	62.0	65.2	67.0	76.2	91.4
Hydrolysis, %	50.2	57.6	62.2	65.5	69.0	71.8	80.5	96.7
K, 2d order const. × 10^3	20	18	17	15	15	15	20	...

$k/4a_{\pm} = 0.023$. Average of constants = 17×10^{-3}.

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[5] D. D. Van Slyke, *J. Biol. Chem.*, **12**, 275 (1912).

TABLE II (*Concluded*)

1.0 *N* Sulfuric Acid

Time, hours	0.5	0.75	1.0	1.25	1.5	1.75	2.0
Amino N per g. of casein, mg.	30.0	37.5	43.8	49.4	52.6	55.1	58.2
Hydrolysis, %	31.8	39.7	46.3	52.2	55.6	58.2	61.5
K, 2d order const. $\times 10^3$	9.4[a]	8.8	8.6	8.7	8.2	8.0	8.0

[a] Value not used in obtaining average.

$k/4a_{\pm} = 0.023$. Average of constants $= 8.4 \times 10^{-3}$.

0.5 *N* Sulfuric Acid

Time, hours	0.5	0.75	1.0	1.25	1.5	1.75	2.0
Amino N per g. of casein, mg.	16.9	23.2	28.8	33.2	40.0	45.7	49.4
Hydrolysis, %	17.8	24.5	30.4	35.0	42.3	48.3	52.2
K, 2d order const. $\times 10^3$	4.3	4.3	4.4	4.3	4.9	5.3	5.4

$k/4a_{\pm} = 0.021$. Average of constants $= 4.7 \times 10^{-3}$.

TABLE III

THE HYDROLYSIS OF CASEIN BY HYDROGEN PHOSPHATE AT 127.5°

2.5 *N* Phosphoric Acid

Time, hours	0.5	0.75	1.0	1.25	1.5	1.75	2.0
Amino N per g. of casein, mg.	16.3	19.4	24.4	26.2	27.5	31.2	33.8
Hydrolysis, %	17.2	20.5	25.8	27.8	29.1	33.1	35.7
K, 2d order const. $\times 10^3$	4.2[a]	3.4	3.5	3.1	2.7	2.8	2.8

$k/a_{\pm} = 0.024$. Average of constants $= 3 \times 10^{-3}$.

3.0 *N* Phosphoric Acid

Time, hours	0.5	0.75	1.0	1.25	1.5	1.75	2.0
Amino N per g. of casein, mg.	18.1	21.9	23.1	25.0	28.8	30.7	35.7
Hydrolysis, %	19.2	23.2	24.5	26.4	30.4	32.5	37.7
K, 2d order const. $\times 10^3$	4.7[a]	4.0	3.3	2.9	2.9	2.8	3.0

[a] Value not used in obtaining average.

$k/a_{\pm} = 0.023$. Average of constants $= 3.2 \times 10^{-3}$.

TABLE IV

THE HYDROLYSIS OF CASEIN BY ACID AT 117.5°

1.0 *N* Hydrochloric Acid

Time, hours	0.5	0.75	1.0	1.25	1.5	2.0
Amino N per g. of casein, mg.	24.4	32.5	38.8	41.9	46.3	52.5
Hydrolysis, %	25.8	34.4	41.0	44.3	48.9	55.5
K, 2d order const. $\times 10^3$	7.0	7.0	7.0	6.4	6.2	6.2

$k/a_{\pm} = 0.0080$. Average of constants $= 6.6 \times 10^{-3}$.

Time, hours	0.5	1.0	1.5	2.0	2.5
Amino N per g. of casein, mg.	25.0	38.8	51.8	56.2	60.4
Hydrolysis, %	26.4	41.0	54.8	59.4	63.4
K, 2d order const. $\times 10^3$	7.2	7.0	8.1	7.3	7.0

$k/a_{\pm} = 0.0089$. Average of constants $= 7.3 \times 10^{-3}$.

TABLE IV (*Concluded*)

1.0 N Sulfuric Acid

Time, hours	0.5	1.0	1.5	2.0	2.5
Amino N per g. of casein, mg.	16.9	26.3	33.8	38.8	44.4
Hydrolysis, %	17.9	27.8	35.7	41.0	46.9
K, 2d order const. × 10^3	4.4[a]	3.9	3.7	3.5	3.5

[a] Value not used in obtaining average.

$k/4a_{\pm} = 0.010$. Average of constants $= 3.6 \times 10^{-3}$.

TABLE V

HYDROLYSIS OF CASEIN BY ACID AT 105.5°

1.0 N Hydrochloric Acid

Time, hours	1	1.5	2.0	2.5	3.0
Amino N per g. of casein, mg.	25.0	32.6	38.2	43.8	46.3
Hydrolysis, %	26.4	34.4	40.3	46.2	48.9
K, 2d order constant × 10^3	3.6	3.5	3.4	3.5	3.2

$k/a_{\pm} = 0.0041$. Average of constants $= 3.4 \times 10^{-3}$.

1.0 N Sulfuric Acid

Time, hours	1	1.5	2.0	2.5	3.0
Amino N per g. of casein, mg.	13.7	19.4	24.4	28.1	31.3
Hydrolysis, %	14.6	20.5	25.8	29.7	33.1
K, 2d order constant × 10^3	1.70	1.72	1.74	1.69	1.65

$k/4a_{\pm} = 0.0048$. Average of constants $= 1.7 \times 10^{-3}$.

Discussion

Order of the Reaction.—The constants of the order of the reactions are calculated, as in the former paper,[1] on the basis of the percentage of hydrolysis according to the formula

$$K = \frac{1}{t} \times \frac{X}{100 - x} \times \frac{1}{100} \qquad (1)$$

in which x represents the percentage of protein hydrolyzed in time t. The constants calculated according to this formula are in fair agreement with each other for each experimental condition of temperature and acid concentration. The reproducibility that can be expected for these experiments is shown in Table IV, in which are given the results of two experiments at the same temperature and acid concentration carried out at different times. The average results of the calculated constants of the two experiments agree within 10% of each other.

That the experimental results best fit the equation for a second order reaction is shown in Table I, in the experiment with 1.0 N hydrogen chloride. In the tabulated results for this experiment there are given the reaction constants as calculated for a first, second and third order reaction It is to be seen that the first order constants regularly decrease in magnitude with time, while the constants for a third order reaction regularly increase in magnitude. This, in conjunction with the agreement of the second order constants, strongly favors the viewpoint that the hydrolysis of casein, as well as of gelatin, silk fibroin and wheat gliadin follows a second order reaction.

Catalysis by Acids.—In agreement with the previous results, the catalytic effect of acids on the hydrolysis of casein has been found to be proportional to the thermodynamic activity of the acids. The velocity constants at unit activity which are given at the foot of each column of velocity constants show good agreement among themselves at each temperature. In making the calculations, the values for the activity coefficients of hydrogen chloride and sulfate were taken from the tables of Lewis and Randall.[6] As a more rigid test of this relationship, two experiments were made using the comparatively weak acid, phosphoric. The unit activity constants obtained are in good agreement with those of sulfuric and hydrochloric acids. As we found no available data for activity coefficients of phosphoric acid in the concentrations employed, measurements of the hydrogen-ion activity of the solutions were made on the hydrogen electrode, using a 0.1 molal calomel hydrogen electrode and bridging across with a saturated KCl bridge. These measurements gave an activity of 0.137 molal for the 3.0 N and 0.123 molal for the 2.5 N acids.

Effect of Temperature.—As in the previous work[1] the mathematical relationship for the change of velocity constants with temperature was determined and the equation obtained by the same procedure as previously described is

$$\log Ka = 0.0307T - 5.61 \qquad (2)$$

in which Ka is the reaction rate constant at unit activity and the temperature T is in degrees centigrade. This gives the means of predicting the course of casein hydrolysis at a given temperature and acid of known activity. The constant, Ka, at any temperature can be obtained from Equation 2. This multiplied by the activity of the acid used gives the velocity constant for that particular acid concentration. The time required to effect a given degree of hydrolysis can be calculated by the use of Equation 1.

Summary

1. The rate of hydrolysis of casein by acids as measured by the increase in amino nitrogen has been found to conform to the equation for a second order reaction.

2. As was previously found for other proteins, the catalytic effect of acids on casein hydrolysis is proportional to the hydrogen-ion activity of the acids.

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[6] Lewis and Randall, "Thermodynamics and the Free Energy of Chemical Substances," McGraw Hill Book Company, New York, **1923**, pp. 336, 357.

3. A quantitative relationship has been derived for the change of the rate of hydrolysis with temperature.

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[CONTRIBUTION FROM THE 2. STATE UNIVERSITY, MOSCOW]

THE MECHANISM OF FORMATION OF BETA-ARYL-BETA-AMINO FATTY ACIDS BY THE CONDENSATION OF AROMATIC ALDEHYDES WITH MALONIC ACID AND ITS DERIVATIVES[1]

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RECEIVED OCTOBER 11, 1928 PUBLISHED MARCH 6, 1929

In our former investigations[2] we have found that the mechanism of Knoevenagel's synthesis of cinnamic acids[3] in the presence of ammonia is much more complicated than Knoevenagel himself and other authors assumed. We have discovered that besides cinnamic acid derivatives the corresponding amino acid is formed with a good yield, in accordance with the following equation

$$RCHO + NH_3 + CH_2(COOH)_2 = RCH(NH_2)CH_2COOH + CO_2 + H_2O$$

By replacing malonic acid by its esters, A. M. Fedorova[2b] obtained the esters of β-aryl-β-amino-isosuccinic acid

$$RCHO + NH_3 + CH_2(COOC_2H_5)_2 = RCH(NH_2)CH(COOC_2H_5)_2 + H_2O$$

After saponification with hydrochloric acid these derivatives give, quantitatively, the corresponding amino acids.

Two possibilities may be worthy of consideration in order to interpret the mechanism of this reaction

I (a) $RCHO + NH_3 = RCH(OH)NH_2$
(b) $RCH(OH)NH_2 + CH_2(COOH)_2 = RCH(NH_2)CH(COOH)_2 + H_2O$
(c) $RCHNH_2CH(COOH)_2 = RCH(NH_2)CH_2COOH + CO_2$
II (a) $RCHO + CH_2(COOH)_2 = RCH{=}CHCOOH + CO_2 + H_2O$
(The final result of Knoevenagel's reaction)
(b) $RCH{=}CHCOOH + NH_3 = RCHNH_2CH_2COOH$

In favor of the second explanation and against the first may be cited the interesting investigations of Körner and Menozzi,[4] Engel[5] and of

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[1] This paper is an abstract of a thesis presented by E. A. Postovskaja in partial fulfilment of the requirements for the degree of Diplom-chemist of the 2. State University of Moscow.

[2] (a) W. M. Rodionow and E. Th. Malevinskaja, *Ber.*, **59**, 2952 (1926); (b) W. M. Rodionow and A. M. Fedorova, *ibid.*, **60**, 804 (1927); (c) *Arch. Pharm.*, **266**, 116–311 (1928).

[3] Knoevenagel, *Ber.*, **31**, 2596 (1898).

[4] Körner and Menozzi, *Ber.*, **21**, ref. 86 (1886); *ibid.*, **22**, ref. 735 (1889); *ibid.*, **27**, ref. 121 (1894).

[5] Engel, *Compt. rend.*, **104**, 1805 (1887); **106**, 1677 (1888).