Physico-chemical Investigation on the Casein-splitting Action of Papain with Special Reference to the Fundamental Properties of its Action as well as to the Mechanism of the Acceleration of HCH Solution upon its Action

By

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(A) Introduction

Concerning the proteolytic activity of papain, a great many investigations have been carried out from various directions. But, here, only the most important literature concerning the present investigation will be briefly mentioned.

(I) On the accelerating action of HCN on the proteolytic activity of papain

This accelerating action of HCN was first observed and discussed by Vines,[1] contrasted with the retarding action of other antiseptics such as toluene, chloroform, sodium fluoride, etc.

According to him, HCN has a great accelerating action not only upon the formation of albumoses and peptones, but also upon the formation of amino acids in its proteolytic action.

Mendel and Blood[2]* carried out further experiments on this subject and obtained the same results as those of Vines.

* Notice:— In their experiments, Witte's peptone, raw and coagulated egg-white, fibrin, edestine, and excelsin, etc., were used as the substrate and the filtrate of 1% NaCl solution of sun-dried powder as enzyme and the intensity of the decomposition of proteins was tested by the deepness of the colour reaction of tryptophane as well as by the quantity of the proteins digested.

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According to them, this accelerating action of HCN could not be attributed,

(a) to the concentration of H-ions;
(b) to the destruction of inhibitory substances in the papain;
(c) to the destruction of inhibitory substances in the substrate;
(d) to the denaturation of substrate;
(e) to the activation of a papain zymogen.

They also carried out further experiments on the influence of some CN-compounds related to HCN on the proteolytic activity of papain and compared with that of HCN.

According to that result, methyl cyanide and potassium sulphocyanide had not a perceptible accelerating action and it was only KCN which had a resembling accelerating action to that of HCN.

Since the above investigation had been carried out, this special action of HCN was verified by D.S. Pratt (1915)[3] and by Edward M. Fränkel (1917).[4] The latter investigator has also shown that it seemed rather difficult to definine a hydrogen ion optimum for papain-HCN proteolysis, the enzyme in the pressence of HCN being equally active, or nearly so, over a wide range of acidity.

Quite recently, Willstätter and Grassmann[5] carried out an investigation in the activation of papain by HCN, using Merck's papain as enzyme and gelatine solution as substrate. The method adopted by them for the determination of the proteolytic activity was "Die Titration der Peptide und Amino-säuren in alkoholischer Lösung nach R. Willstätter und E. Waldschmidt-Leitz."

According to the results obtained by them, the activity-PH-curves for papain and papain-HCN (Papain previously treated with HCN for 2 hours) were noticeably different, and in this case, unlike the result obtained by Fränkel, there was also a definite H-ion optimum for papain-HCN proteolysis, such as had been determined for papain alone.

(II) On the influence of acids, bases, and salts upon the proteolytic activity of papain with special reference to the influence of hydrogen ion concentration

According to A. Baginski (1883),[6] the optimum reaction for the proteolytic activity of papain is neutral, while, according to O. Emmerling (1902),[7] J. E. Rippetoe (1912),[8] etc., it is weak alkaline, and according to H. T. Graber (1910),[9] it is weak acidic.

Pratt (l.c.) investigated on the proteolytic activity of papain, using

milk protein as substrate and papain prepared by him as enzyme and obtained the following results:—

(a) HCl up to 0.06% has only a slight retarding action on the digestion of milk protein; increasing amounts, from 0.06 to 0.13%, acid very greatly reduces the activity of the enzyme, although a further increase up to 0.2% causes practically no change.

(b) $NaHCO_3$ in amounts ranging from 0.0 to 0.2% has no effect on the digestion of milk protein; similar negative results were also obtained with concentrations of NaCl varying from 0.1 to 1.0%.

Fränkel (l.c) carried out an experiment, using casein as substrate and papain purified by him as enzyme.

According to his result, papain exhibits its greatest activity in a slight acid solution with a H-ion concentration of 10^{-5}. The enzyme is sensitive to both, acid and alkali, but alkali has a less destructive action.

Chestnut (1920)[10] carried out detailed experiments on many samples of papain latex prepared in various ways from all available varieties of fruit in all stages of growth, using casein as substrate and measuring the intensity of the proteolytic activity by the polariscopic method. According to his results:—

(a) The optimum medium for the proteolytic activity of papain was one prepared from 25 cc. of 4% alkaline casein solution, 2.5 cc. of N/5 HCl and 17.5 cc. of water, its H-ion concentration being PH= 6.21.*

(b) The following curves (Fig. I) plotted for pepsin, trypsin, and papain show the influence of PH on the activity of these enzymes.

Brill and Brown (1920)[11] carried out an experiment on papain, using a 10% solution of skimmed milk powder as substrate. The following results were obtained.

(a) NaCl, KCl, and Na-citrate slightly increase the activity;

(b) Na_2CO_3, $NaHCO_3$, $CaCl_2$, $MgSO_4$, and H_3BO_3 are without effect;

(c) Acetic acid and lactic acid strongly inhibit the activity.

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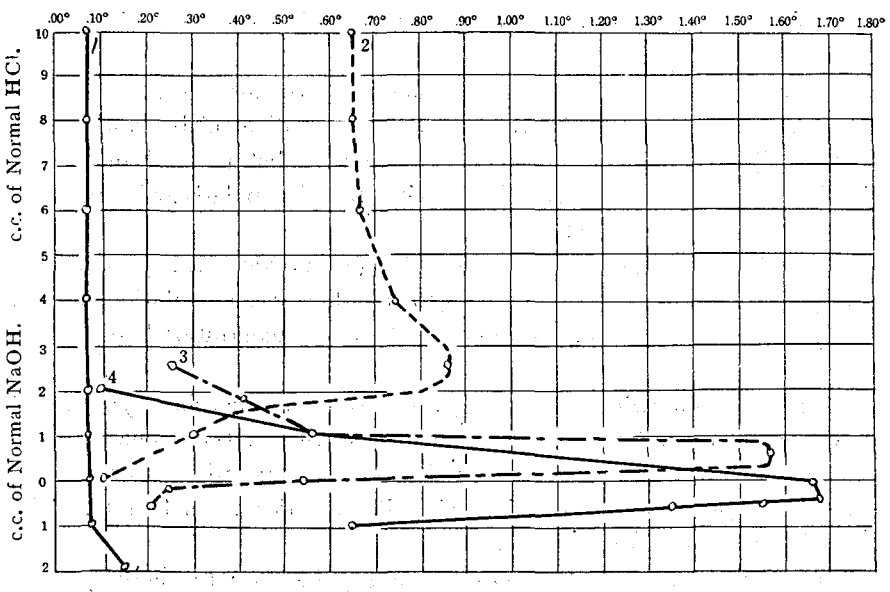
* Notice:— (i) 25 cc. of that alkaline casein solution, to which 20 cc. of water were added, had a hydrogen ion concentration of PH = $10^{-9.35}$.

(ii) In the digestion experiment, 5 cc. of enzyme were added to that medium, making each total volume 50 cc. The Extent of activity varied only a little even if 2–4 cc. of N/5 HCl were used, but the proteolytic activity of papain dropped off rapidly if less than 2 cc. were used and less rapidly if more than 4 cc. were used.

(III) On the influence of temperature upon the proteolytic activity of papain

According to Delezenne, Mouton, and Pozerski (1906),[12] at high temperatures, papain digests the proteins of egg-white and serum, so rapidly, into albumoses and peptones. That is, when a suitable mixture of enzyme and uncoagulated proteins slightly acidified, is heated quickly to boiling over a free frame, scarcely any coagulum is obtained, though almost all the proteins dissolve altogether.

Fig. I.



Pratt (l.c.) carried out experiments on the proteolytic activity of papain at various temperatures ranging from 0°. to 70°, using milk protein as substrate and allowing to digest for 30 minutes. According to these results :—

(a) Papain shows a remarkable activity even at low temperatures.

(b) There appears to be very little difference in the rate of digestion at the temperature from 40° to 50°.

(c) The digestion at a high temperature as 70°, shows that the activity in the presence of large amounts of the enzyme is not greatly weakened, but with decreasing percentages of papain, it becomes markedly weak.

(d) A papain solution, rapidly heated to 100°, left for 5 seconds and immediately cooled with ice, no longer shows any proteolytic activity.

According to Pozerski (1917),[13] the optimum temperature for the proteolytic activity of papain is very high, generally very near that of its destruction.

According to Mendel and Blood (l.c.), protein of egg-white as well as typical plant proteins such as excelsin and edestin, are digested very rapidly at 80°.

According to Hagiwara (1924),[14] the optimum temperature for the proteolytic activity of papain latex is 75°–90°. At 95°, the activity of papain decreases very markedly.*

(IV) On the question of the formation of amino acids by papain in its proteolytic action.

On this question, many investigations had also been carried out by various authors, but the results obtained, did not, on the whole, agree with one another.

By some authors, (Wurtz,[15] Martin,[16] Vines,[17] etc.,) a perceptible quantity of amino acids could be obtained in the digestion products of proteins by papain, while, by others, (Chittenden,[18] Sharp,[19] Dott,[20] Chittenden and Mendel and Mc. Dermott,[21] Harlay,[22] Mendel and Underhill,[23] Emmerling,[24] Kutscher and Lohmann,[25] Abderhalden and Terauchi,[25] etc.,) either no perceptible quantity of amino acids could be thus obtained in the ordinary case or it was very small even when a large proportion of papain was used and the digestion was prolonged for a long time, as 6 weeks, 10 months, etc.

According to Vines (1905),[27] these discrepancies were due to the use of different antiseptics by different investigators. That is, positive results were obtained by the authors who had used HCN as an antiseptic, though no positive results could be obtained by the authors who had used common antiseptics such as toluene, chloroform, etc.

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* Notice:— The proteolytic activity of papain was determined by the method adopted by Pratt.

According to Mendel and Blood (1910),[28] Pratt (l.c.), Fränkel (l.c.), etc., HCN has a great accelerating action not only upon the formation of albumoses and peptones, but also upon the formation of amino acids in its proteolytic action.

According to Delaunay and Bailly (1913),[29] papain is shown to be a peptone forming, rather than a peptolytic enzyme.

According to Deleanu (1915–1916),[30] various vegetable proteins when heated rapidly with papain are hydrolysed and yield proteoses, peptones and amino acids. However the intensity of forming amino acids is different according to the kinds of proteins.

As will be seen from the review stated above, on the proteolytic action of papain, no investigation appears to have been carried out sufficiently from the point of view of physico-chemistry and there are also not a few results which did not, on the whole, agree with one another.

Therefore, on the one hand, in order to give some trustworthy verification to those questionable results, and on the other hand, in order to give some further clear explanation on the mechanism of the acceleration of HCN solution upon the proteolytic action of papain, the present investigation has been carried out, as the author thought it to be most important from the point of view of enzyme chemistry.

The author would like to tender his cordial thanks to Prof. Dr. K. Oshima, Director of the College of Science and Agriculture in the Taihoku Impeaial University, who has given the author invaluable advice during the course of his investigation and hearty thanks are also due to Prof. Dr. K. Miyake, Prof. Dr. S. Itō and Prof. Dr. T. Tadokoro in the Hokkaido Imperial University, who have given their thoughtful suggestions during the course of preparation of this paper.

(B) On the Fundamental Properties of the Casein-splitting Action of Papain

(I) On the methods for the quantitative determination of proteolytic activity

On the methods for the quantitative determination of the proteolytic activity, many examinations have been carried out by various authors. According to Sherman and Neun,[31] of all the methods described below, the method by the quantitative determination of total nitrogen or the amino nitrogen of the digestion products (or both) is considered intensely delicate and very accurate.

The methods studied in their examination were :—

(1) by the determination of total digested nitrogen by Kjeldahl's method,

(2) by the determination of amino nitrogen as measured by the Van Slyde's method, (They have accepted the results of Harding and Mc.Lean as sufficiently indicative that the results of the Sörensen's method run parallel with those of the Van Slyke's method and so have not included the Sörensen's method in their comparative examination.)

(3) by the acidmetric titration of digestion products,

(4) by the determination of the change in electrical conductivity.

(5) by the determination of the change in rotation of polarized light,

(6) by the Mett's method,

(7) by the determination of the depth in the biuret reaction,

(8) by the determination of the depth in the ninhydrin reaction.

The preliminary experiment which has been carried out by the author led to the same conclusion as Sherman and Neun. Therefore, in all the following experiments, the methods by the determination of total digested nitrogen as well as of amino nitrogen as measured by the Sörensen's method were adopted. Merck's casein (nach Hammarsten) of best quality was used as substrate and papain used was one prepared and purified by the author by the following way. The detailed process of the experiments will be described in each experimental part.

At Heito, Formosa, the author punctured, with a wood spatula, the rind of fully developed but unripe green fruits of papaya trees. The white milky latex which gushes from the rind, was collected into an evaporating porcelain besin; then it was precipitated by adding 95% alcohol, and filtered immediately. The precipitate was placed into a bottle after washing it with ether, and carried into author's chemical laboratory in the Government College of Agriculture and Forestry at Taihoku and dried over sulphuric acid in a vacum desiccator, white powder being thus obtained. The purification of the white powder was then carried out as follows :—

The white powder was extracted with water for several times and filtered. The filtrate was concentrated into about 1 L. by the vacum evaporation at 40° C. To 1 volume of the concentrated filtrate were added 5 volumes of 95% alcohol and the precipitate thus formed was filtered immediately. The precipitate was dissolved in as small a quantity of water as possible and precipitated again with 95% alcohol and the precipitate was filtered immediately.

This process of dissolving, precipitation and filtration, was repeated once more. The precipitate thus finally obtained was washed well with ether and dried in a vacuum desiccator. This was used, as the purified papain, in the following experiments.

(II) On Casein-splitting Action by the various Amounts of Papain.

According to one of the laws of catalytic action in the homogeneous system. The reaction velocity in other words the velocity constant, is proportional to the quantity of catalyser, provided that the concentration of the substrate remains constant.[32]

As to the law in the case of enzymes, various conflicting statements have been made.

Some observers have found that the reaction velocity is proportional to the quantity of enzyme, but others have found that it is not proportional and the specific activity, i., e., the activity per unit amount of enzyme is less in the case of high concentration of enzyme than in the case of lower ones.[33]

Nelson and Vosburgh[34] investigated on this relation with invertase and Van Slyke and Cullen with ureas and found that, in each case, the reaction velocity was proportional to the quantity of enzyme.

Hedin (1905),[35] found that, in the case of trypsin, similar relationship as above was obtained, when the substrate was in excess.

E. Schütz and Borissov (1885)[36] have gone so far as to formulate a law, according to which, the quantity of substrate digested by pepsin is proportional to the square root of the product of the quantity of enzyme and the time of digestion, as the following formula shows :—

$$X=K\sqrt{PT}$$

where, X=the quantity of substrate digested by the quantity P of pepsin during the time T.

K=constant.

In the case of castor oil lipase, as investigated by Jalander (1911),[37] the square root law is only approximately obeyed.

Kjeldahl (1879)[38] had already published curves and figures showing this variation of the ratio of enzyme concentration to the activity in different stages of the reaction, viz., first, linear ; then exponential ; and finally, again linear. He also showed that the curve of the velocity of reaction had the same form when the enzyme and the substrate were in a certain proportion to one another.

According to Bayliss (1919),[39] these discrepant rules may all be

correct, but that they apply to different relative concentrations of enzyme and substrate, or, in other words, to different stages of reaction, when this begins with relative excess of substrate.

When the enzyme is in considerably smaller concentration than the substrate, the velocity of the reaction is in direct linear proportion to the quantity of enzyme present, as the whole of it enters into effective combination with substrate.

As the concentration of the substrate diminishes, another law begins to make its appearance, so that the greater quantities of enzyme have relatively less effect.

The so called "law" of Schütz and Borissov is one particular case of this relationship.

In his experiment, trypsin was used as enzyme and 2.5% solution of ammonium caseinate as substrate and the results above stated were obtained by the determination of the change in the electrical conductivity.

Philoche (1908),[40] in an exhaustive work on amylase and maltase, gives an empirical formula to express the relation of the activity of these enzymes to their concentration as follows:—

$$X=BC-AC^2$$

where, X being the activity, C the concentration of enzyme, B and A constants. This is practically a case of the general exponential law of absorption.

The general review of the investigations which have been carried out on the relationship of the activities of various kinds of enzymes to their quantities is as above stated. In the case of papain, this relationship does not seem to have been fully investigated. Therefore, the author carried out the following experiment on the casein-splitting action with various amounts of papain.

Experimental process

(a) Preparation of 2% casein solution.

Ten g. of casein (calculated as water free substance) were taken into a 1 L. porcelain basin and to it were added 300 cc. of distilled water and 20 cc. of N/1 NaOH solution. The mixture was kept on a water bath at a temperature of 70°–80°, with constant stirring, until it became a clear solution, and then, cooled and neutralized with N/10 HCl solution, using phenolphthalein as indicator and made up to 500 cc. with water.

(b) Preparation of 0.05% papain solution.

0.05 g. of papain were dissolved in 50 cc. of water by keeping it at 40° for 30 minutes in a thermostat, and then, made up to 100 cc. with water after cooling. Thus a fairly clear solution was obtained, but in the present experiment, it was used after filtration.

(c) Process of digestion

Twenty five cc. of 2% casein solution were placed in a series of 300 cc. Erlenmeyer's flasks and to them were added, various amounts of 0.05% papain solution and water as in the Table (I), making each total volume 150 cc. The mixture in each flask was, then, allowed to digest for 2 hours at 40° in a constant temperature bath. In order to prevent evaporation of water, each flask was corked with a cork in which was inserted a long glass tube.

On the other hand, 5 cc. of N/1 HCl solution and 25 cc. of 20% Na_2SO_4 solution were prepared, as precipitant, in a series of beakers.* When the process of digestion was finished, the mixture in each flask was poured off into each beaker prepared as above in order to stop the action of enzyme as well as to precipitate the casein undigested. The precipitate of the undigested casein was washed with 5% Na_2SO_4 solution and the total nitrogen of which was determined by the Kjeldahl's method.

The precipitate was introduced into a 500 cc. Kjdldahl's flask, mixed with 10 cc. of concentrated H_2SO_4 salution, 1 drop of mercury, and boiled for several hours till it was thoroughly decomposed. Then, the flask was allowed to cool; after which the content was carefully diluted with water, making each total volume up to 250 cc. and 100 cc. of which were put into a distillation flask for the determination of the nitrogen. In each receiver of the distillate, 20 cc. of N/5 H_2SO_4 solution were placed and the N/5 H_2SO_4 solution partly neutralized in each receiver was titrated by means of N/5 NaOH solution, using congo red as indicator and from the results of which, cc. of N/5 NaOH solution corresponding to the casein-N digested was calculated.

Experimental results

Results obtained from the above experiment are given in the Table (I).

* According to the preliminary examination, 5 cc. of N/1 HCl solution and 25 cc. of 20% Na_2SO_4 solution were sufficient to stop the action of 50 cc. 0.1% papain solution.

TABLE (I)

The quantities of casein *N* digested by various amounts of papain.
The length of time of digestion = 2 hr. + 15 min.
The temperature of digestion = 40°C.

No.	c.c. of 2% casein solution	c.c. of 0.05% papain solution	c.c. of H_2O	c.c. of *N*/5 NaOH solution titrated (=a)	c.c. of *N*/5 NaOH solution corresponding to the total casein-N undigested (A=(20−a) 2.5)	c.c. of N/5 NaOH solution corresponding to the total casein-N digested (19.250−A)
1	25	0 boiled	125	12.30	19.250	0
2	25	10	115	12.30	19.250	0
3	25	1	124	15.95	17.625	1.625
4	25	2	123	13.60	16.000	3.250
5	25	3	122	14.20	14.500	4.750
6	25	4	121	14.80	13.000	6.250
7	25	5	120	15.45	11.375	7.875
8	25	6	119	16.05	9.875	9.375
9	25	7	118	16.50	8.750	10.500
10	25	8	117	16.95	7.625	11.625
11	25	9	116	17.35	6.625	12.625
12	25	10	115	17.75	5.625	13.625
13	25	15	110	18,70	3.250	16.000
14	25	20	105	19.05	2.375	16.875
15	25	30	95	19.20	2.000	17.250
16	25	40	85	19.25	1.875	17.375
17	25	50	75	19.25	1.875	17.475
18	25	100	25	19.50	1.250	18.000

From the results it is seen that in the case of digestion at 40°, using 25 cc. of 2% casein solution as substrate, the reaction velocity (the quantity of casein digested during 2 houre in this case) is directly proportional to the concentration of papain, provided that 0.05% papain solution is used in a range from 1 cc. to 10 cc.

However, in the case of using more than 10 cc. of 0.05% papain solution, the reaction velocity is not proportional to the concentration of papin; and though the papin solution is ever increased, the increasing degree of that reaction velocity becomes gradually less, and finally

reaches its equilibrium condition, the reaction velocity remaining always constant, without increasing any more.

The reason of why the increasing degree of the reaction velocity diminishes, while the quantity of papain solution used increases, is probably due to the fact that, although the reaction volocity of the one had reached its equilibrium condition at first, and stopped, the reaction velocity of the other still proceeds without reaching its equilibrium condition and consequently, the latter approaches the former more closely. The Table (II) shows the deviation of constant K calculated from the formula of the Schütz's law.

TABLE (II)

Constant K calculated from the Schültz's formula $X=K\sqrt{P.\ t.}$
(log. K=log. $X-1/2$ log. $P-1/2$ log. t).

No.	Time (hr.)	P	X	Log. K	K
1	0	0	0	0	0
2	2	1	1.625	0.06034	1.14905
3	2	2	3.250	0.21085	1.62500
4	2	3	4.750	0.28762	1.93918
5	2	4	6.250	0.34434	2.20974
6	2	5	7.875	0.39625	2.49029
7	2	6	9.375	0.43238	2.70631
8	2	7	10.500	0.44813	2.80627
9	2	8	11.625	0.46334	2.90627
10	2	9	12.625	0.47360	2.97578
11	2	10	13.625	0.48382	3.04664
12	2	15	16.000	0.46556	2.92120
13	2	20	16.875	0.42622	2.66819
14	2	30	17.250	0.34772	2.22700
15	2	40	17.375	0.28828	1.94214
16	2	50	17.375	0.23982	1.73708
17	2	100	18.000	0.10476	1.27279

(III). On the Reaction Velocity in the Casein-splitting Action of Papain with Special Reference to the Velocity Constant

According to one of the laws of catalytic action in the homogeneous system, the reaction velocity at a moment is proportional to the

concentration of the substrate reacting at that moment[41] provided that the quantity of catalyzer remains constant as stated before.

Accordingly, in the case of monomolecular reaction, the rection velocity constant K is to be expressed[42] by either of the following formulae.

$$K_1=(1/t)\ \text{Ln. a}/(a-x),$$
$$\text{i.e., } 0.4343\ K_1=(1/t)\ \text{Log. } a/(c-x)=K \quad \ldots\ldots\ldots\ldots\ (1)$$
$$K_2=[1/(t_2=t_1)]\ \text{Ln. } C_1/C_2,$$
$$\text{i.e., } 0.4343\ K_2=[1/(t_2-t_1)]\ \text{Log. } C_1/C_2,=K \quad \ldots\ldots\ldots\ (2)$$

Where, t, t_1, and t_2=the times which have elasped since the beginning of the reaction, a=the initial concentration of the substrate, x=the amount of the substrate changed during the time t, C_1 and C_2=the concentrations of the substrate at the times, t_1 and t_2, respectively, and the formula (2) was derived from the formula (1).

However, in the general case of the catalytic action by enzyme, the law of mass action is not strictly applicable and the reaction velocity is not proportional to the concentration of the substrate reacting at that moment, even when the quantity of catalyzer remains constant.

Consequently, the constant K in the above formulae gradually increases or decreases[43] as the reaction proceeds.

For example, V. Henri (1903) obtained a steady rise on invertase, Frankland Armstrong (1919), a steady fall on lactase, and Bayliss, a marked diminution on trypsin, in the values of constant K, while, on papain, no experimental result to show the values of constant K seems to have been obtained. Therefore, the author carried out the following experiment on this subject.

Experimental process

Twenty five cc. of 2% casein solution were placed in a series of 300 cc. Erlenmeyer's flasks and to them were added definite amounts of 0.05% papain solution and water as in the Tables, (III) (A), (III) (B), (III) (C), making each total volume 150 cc. The mixture in each flask was allowed to digest at 40° in a constant temperature bath, during various lengths of time* as in the table above stated. The

* The temperature of the contents in each Erlenmyer's flask reached 40°C within 15 minutes in the water bath at 40°C. So this point was taken as a standard of measuring the lengths of time of digestion. This relationship was also the same in the following various tables.

further detailed process is just the same as in the case of the experiment (11).

Experimental results

Results obtained from the above experiments are given in the Tables, (III) (A), (III) (B), (III) (C), and illustrated in curves in Fig. (II).

TABLES, (III) (A), (III) (B), (III) (C)

The quantities of casein *N* digested by papain during various lengths of time.

(III) (A). In the case when 3 cc. of 0.05% papain solution were used.

No.	Time of digestion (min.)	c.c. of 2 % casein solution	c.c. of 0.05 % of papain solution	c.c. of H_2O	c.c. of *N*/5 NaOH solution titrated(=*a*)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* undigested ($N=(20-a)2.5$)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* undigested ($19.375-A$)
1	Control.	25	0	125	12.25	19.375	0
2	15	25	3	122	12.70	18.250	1.125
3	20+15	25	3	122	13.00	17.500	1.875
4	40+15	25	3	122	13.30	16.750	2.625
5	60+15	25	3	122	13.60	16.000	3.375
6	80+15	25	3	122	13.90	15.250	4.125
7	100+15	25	3	122	14.20	14.500	4.875
8	120+15	25	3	122	14.50	13.750	5.625
9	140+15	25	3	122	14.80	13.000	6.375
10	160+15	25	3	122	15.10	12.250	7.125
11	180+15	25	3	122	15.40	11.500	7.875
12	200+15	25	3	122	15.70	10.750	8.625
13	220+15	25	3	122	16.00	10.000	9.375
14	240+15	25	3	122	16.30	9.250	10.125
15	260+15	25	3	122	16.60	8.500	10.875
16	280+15	25	3	122	16.80	8.000	11.375
17	300+15	25	3	122	16.90	7.750	11.625

TABLE (III) (B)

In the case when 5 cc. of 0.05% papain solution were used.

No.	Time of digestion (min.)	c.c. of 2 % casein solution	c.c. of 0.05 % papain solution	c.c. of H_2O	c.c. of $N/5$ NaOH solution titrated(=a)	c.c. of $N/5$ NaOH solution corresponding to the total casein-N undigested ($A=(20-a)2.5$)	c.c. of $N/5$ NaOH solution corresponding to the total casein-N digested ($19.375-A$)
1	Control.	25	0	125	12.25	19.375	0
2	15	25	5	120	12.80	18.000	1.375
3	20+15	25	5	120	13.25	16.875	2.500
4	40+15	25	5	120	13.70	15.750	3.625
5	60+15	25	5	120	14.15	14.625	4.750
6	80+15	25	5	120	14.60	13.500	5.875
7	100+15	25	5	120	15.05	12.375	7.000
8	120+15	25	5	120	15.50	11.250	8.125
9	140+15	25	5	120	15.95	10.125	9.250
10	160+15	25	5	120	16.40	9.000	10.375
11	180+15	25	5	120	16.85	7.875	11.500
12	200+15	25	5	120	17.30	6.750	12.625
13	220+15	25	5	120	17.65	5.875	13.500
14	240+15	25	5	120	17.90	5.250	14.125
15	260+15	25	5	120	18.10	4.750	14.625
16	280+15	25	5	120	18.30	4.250	15.125
17	300+15	25	5	120	18.40	4.000	15.375

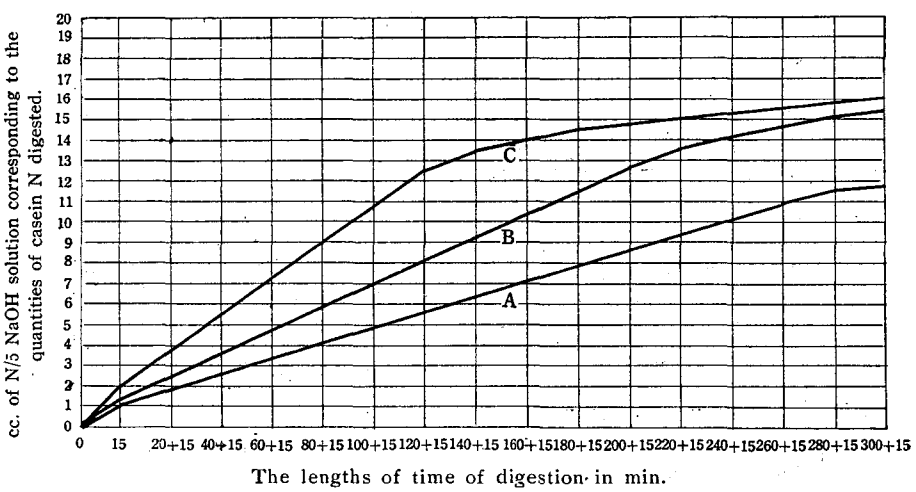
TABLE (III) (C)

In the case when 7 cc. of 0.05% papain solution were used.
The length of time of digestion
=as noted in the following table.
The temperature of digestion=40°C.

No.	Time of digestion (min.)	c.c. of 2 % casein solution	c.c. of 0.05 % papain solution	c.c. of H_2O	c.c. of $N/5$ NaOH solution titrated(=a)	c.c. of $N/5$ NaOH solution corresponding to the total casein-N undigested ($A=(20-a)2.5$)	c.c. of $N/5$ NaOH solution corresponding to the total casein-N digested ($19.375-A$)
1	Control.	25	0	125	12.250	19.375	0
2	15	25	7	118	13.050	17.375	2.000
3	20+15	25	7	118	13.750	15.625	3.750
4	40+15	25	7	118	14.450	13.875	5.500
5	60+15	25	7	118	15.150	12.125	7.250
6	80+15	25	7	118	15.850	10.375	9.000
7	100+12	25	7	118	16.550	8.625	10.750

No.	Time of digestion (min.)	c.c. of 2 % casein solution	c.c. of 0.05 % papain solution	c.c. of H_2O	c.c. of $N/5$ NaOH solution titrated ($=a$)	c.c. of $N/5$ NaOH solution corresponding to the total casein-N undigested ($A=(20-a)2.5$)	c.c. of $N/5$ NaOH solution corresponding to the total casein-N digested ($19.375-A$)
8	120+15	25	7	118	17.250	6.875	12.500
9	140+15	25	7	118	17.650	5.875	13.500
10	160+15	25	7	118	17.850	5.375	14.000
11	180+15	25	7	118	18.050	4.875	14.500
12	200+15	25	7	118	18.150	4.625	14.750
13	220+15	25	7	118	18.250	4.375	15.000
14	240+15	25	7	118	18.350	4.125	15.250
15	260+15	25	7	118	18.450	3.875	15.500
16	280+15	25	7	118	18.550	3.625	15.750
17	300+15	25	7	118	18.650	3.375	16.000

Fig. II.



The results show that in the case of the present experiment, the degree of the curvature of the reaction-velocity-curves is very small and makes nearly straight lines in the main part of the reaction, i.e., during which, the law of mass action is not strictly applicable and the reaction velocity gradually diminishes though slightly, but not in proportion to the concentration of the substrate, which diminishes as the reaetion goes on.

However, it is to be noticed here, that the degree of the curva-

ture of these reaction-velocity-curves is gradually increased, as the reaction goes on near the equillbrium condition.

In the Tables, (IV) (A), (IV) (B), (IV) (C), is calculated from the above result, the deviation of constant K in the formula of monomolecular reaction and is illustrated in curves in Fig. (III).

TARLES, (IV) (A), (IV) (B), (IV) (C)

Constant K calculated from the formula of monomolecular reaction.

$K=0.4343\ K_1=1/t$ log. $a/a-x$.

TABLE (IV) (A)

No.	Time (min.)	a	$a-x$	Log. $a/(a-x)$	$1/t$ Log. $a/(a-x)$ $=0.4343\ K$
1	0	19.375	0	0	0
2	15	19.375	18.250	0.02598	0.00173
3	35	19.375	17.500	0.04420	0.00126
4	55	19.375	16.750	0.06323	0.00115
5	75	19.375	16.000	0.08312	0.00111
6	95	19.375	15.250	0.10397	0.00109
7	115	19.375	14.500	0.12587	0.00109
8	135	19.375	13.750	0.14894	0.00110
9	155	19.375	13.000	0.17330	0.00112
10	175	19.375	12.250	0.19910	0.00114
11	195	19.375	11.500	0.22654	0.00116
12	215	19.375	10.750	0.25583	0.00119
13	235	19.375	10.000	0.28724	0.00122
14	255	19.375	9.250	0.32110	0.00126
15	275	19.375	8.500	0.35782	0.00130
16	295	19.375	8.000	0.38415	0.00130
17	315	19.375	7.750	0.39794	0.00126

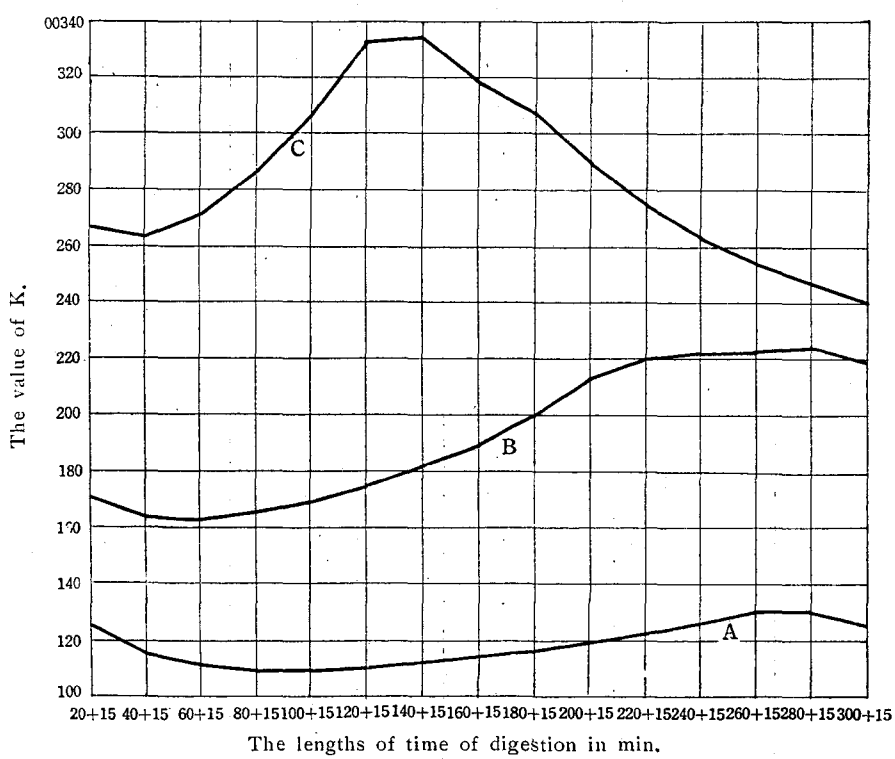
TABLE (IV) (B)

No.	Time (min.)	a	$a-x$	Log. $a/(a-x)$	$1/t$ Log. $a/(a-x)$ $=0.4343\ K$
1	0	19.375	0	0	0
2	15	19.375	18.000	0.03192	0.00213
3	35	19.375	16.875	0.06000	0.00171
4	55	19.375	15.750	0.08996	0.00164
5	75	19.375	14.625	0.12214	0.00163
6	95	19.375	13.500	0.15691	0.00165
7	115	19.375	12.375	0.19470	0.00169
8	135	19.375	11.250	0.23609	0.00175
9	155	19.375	10.125	0.28185	0.00182
10	175	19.375	9.000	0.33300	0.00190
11	195	19 375	7.875	0.39099	0.00200
12	215	19.375	6.750	0.45794	0.00213
13	235	19.375	5.875	0.51823	0.00220
14	255	19.375	5.250	0.56708	0.00222
15	275	19.375	4.750	0.61055	0.00222
16	295	19.375	4.250	0.65885	0.00223
17	315	19.375	4.000	0.68518	0.00218

TABLE (IV) (C)

No.	Time (min.)	a	$a-x$	Log. $a/(a-x)$	$1/t$ Log. $a/(a-x)$ $=0.4343\ K$
1	0	19.375	0	0	0
2	15	19.375	17.375	0.04732	0.00315
3	35	19.375	15.625	0.09342	0.00267
4	55	19.375	13.875	0.14500	0.00264
5	75	19.375	12.125	0.20356	0.00271
6	95	19.375	10.375	0.27125	0.00286
7	115	19.375	8.625	0.35148	0.00306
8	135	19.375	6.875	0.44997	0.00333
9	155	19.375	5.875	0.51823	0.00334
10	175	19.375	5.375	0.55686	0.00318
11	195	19.375	4.875	0.59927	0.00307
12	215	19.375	4.625	0.62213	0.00289
13	235	19.375	4.375	0.64626	0.00275
14	255	19.375	4.125	0.67192	0.00263
15	275	19.375	3.875	0.69897	0.00254
16	295	19.375	3.625	0.72793	0.00247
17	315	19.375	3.375	0.75897	0.00241

Fig. III.



As will be clearly seen from the Fig. (III), the value K corresponding to the constant increases gradually as the reaction proceeds.

(IV) On the Influence of the Concentration of Casein upon the Casein-splitting Action of Papain

In the previous experiment, the author found out that the degree of the curvature of the reaction-velocity-curves of the casein splitting action of papain is very small and makes nearly a straight line in the main part of the reaction, i.e., during which, the law of mass action is not strictly applicable and the reaction velocity gradually diminishes though slightly, but not in proportion to the concentration of the substrate, which diminishes as the reaction goes on.

In ordor to give further verification to this fact, the auther carried out the following experiment on the velocity curves, using casein solutions of various concentrations as substrate from the beginning of the reaction.

Experimental process

In a series of 300 cc. Erlenmeyer's flasks, were placed, as in the Table (V), definite amounts of 2% casein solution, 0.05% papain solution and water, making each total volume 150 cc., and the mixture in each flask was allowed to digest at 40° during various lengths of time as in the table above mentioned. Further detailed process is just the same as in the case of the experiment (II). Tne purpose of this experiment was to compare reaction-velocity-curves in the various concentrations of casein as given by the following three cases :—

(a) 35 cc. of 2% casein solution contained in the total volume 150 cc.

(b) 25 cc. of 2% casein solution contained in the total volume 150 cc.

(c) 15 cc. of 2% casein solution conlained in the total volume 150 cc.

Experimental results

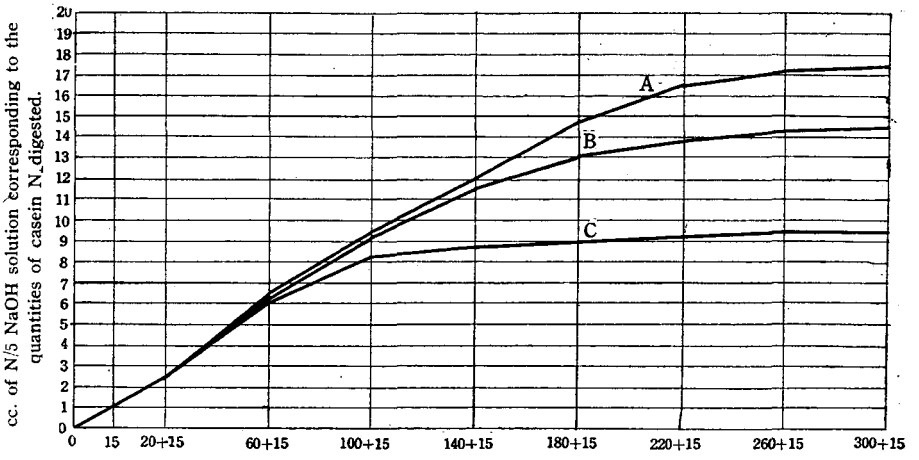
Results obtained from the above experiment are given in the Table (V) and illustrated in curves in Fig. (IV).

TABLE (V)

The quantities of casein *N* digested by papain in various concentrations of casein, during various lengths of time.

No.	Time of digestion (min.)	c.c. of 2 % casein solution	c.c. of 0.05 % papain solution	c.c. of H_2O	c.c. of *N*/5 NaOH solution titrated(=*a*)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* undigested ($A=(20-a)2.5$)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* digested
	Control.						
1	300+15	35	0	115	8.40	29.000	0
2	15	35	5	110	8.80	28.000	1.000
3	20+15	35	5	110	9.40	26.500	2.500
4	60+15	35	5	110	11.00	22.500	6.500
5	100+15	35	5	110	12.20	19.500	9.500
6	140+15	35	5	110	13.20	17.000	12.000
7	180+15	35	5	110	14.30	14.250	14.750
8	220+15	35	5	110	15.00	12.500	16.500
9	260+15	35	5	110	15.30	11.750	17.250
10	300+15	35	5	110	15.40	11.500	17.500
	Control.						
11	300+15	25	0	125	11.70	20.750	0
12	15	25	5	120	12.10	19.750	1.000
13	20+15	25	5	120	12.70	18.250	2.500
14	60+15	25	5	120	14.20	14.500	6.250
15	100+15	25	5	120	15.40	11.500	9.250
16	140+15	25	5	120	16.30	9.250	11.500
17	180+15	25	5	120	16.90	7.750	13.000
18	220+15	25	5	120	17.20	7.000	13.750
19	260+15	25	5	120	17.40	6.500	14.250
20	300+15	25	5	120	17.50	6.250	14.500
	Control.						
21	300+15	15	0	135	15.00	12.500	0
22	15	15	5	130	15.40	11.500	1.000
23	20+15	15	5	130	16.00	10.000	2.500
24	60+15	15	5	130	17.40	6.500	6.000
25	100+15	15	5	130	18.30	4.250	8.250
26	140+15	15	5	130	18.50	3.750	8.750
27	180+15	15	5	130	18.60	3.500	9.000
28	220+15	15	5	130	18.70	3.250	9.250
29	260+15	15	5	130	18.80	3.000	9.500
30	300+15	15	5	130	18.80	3.000	9.500

Fig. IV.



In comparing these three reaction-velocity-curves, solutions of various casein concentrations were used as substrate, from the beginning of the reaction, [a, b, c, in Fig. (IV)], and the author has reached the following conclusion.

In the first stages of each reaction, these three reaction-velocity-curves run very closely with one another, consequently, when these points, in each curve, corresponding to the same value on the abscissa, are compared, it is clearly seen that, in these stages, the reaction velocity in the case of lower casein concentration is less though very slightly than in the case of higher casein concentration.

This fact verifies the conclusion obtained in the previous experiment (III), that the reactioh velocity does gradually increase though very slightly, but not in proportion to the casein concentration.

However, as each reaction approached the later stages, the intervals between these three curves were gradually increased. This is probably due to the fact that, in the case of lower casein concentration such as (c), the reaction reaches its equilibrium condition comparatively quicker than in the case of higher casein concentration auch as (a), its reaction having been stopped; while, in the case of higher casein concentration such as (a), the reaction reaches its equilibrium condition comparatively slower than in the case of lower casein concentration such as (c), its reaction being still continued.

(V) On the Influence of the Temperature of Digestion upon the Casein-spilitting Action of Papain with a Special Reference to the Temperature Coefficients

As a general rule, chemical reactions are increased very markedly by the rise of temperature. With regard to this influence of the temperature upon the chemical reactions, following equations are proposed[44] by Van't Hoff, Arrhenius, D. M. Kooy, respectively :—

$$d \text{ Ln. } K/d\ T = A/T_2 + B \quad \text{..... Van't Hoff} \quad (1)$$
$$d \text{ Ln. } K/d\ T = A/T_2 \quad \text{..... Arrhenius} \quad (2)$$
$$d \text{ Ln. } K/d\ T = (A+BT)/T_2 \quad \text{..... D. M. Kooy} \quad (3)$$

where, K=the velocity constant,
T=the temperature,
A and B=constants.

Integrating above three equations,

$$\text{Ln. } K = -A/T + BT + C \quad \text{.....} \quad (1')$$
$$\text{Ln. } K = -A/T + C \quad \text{.....} \quad (2')$$
$$\text{Ln. } K = A/T + B \text{ Ln. } T + C \quad \text{.....} \quad (3')$$

where, A, B, and C are constants in each formula respectively. The ratio of the velocity constants, K_1 and K_2, at different temperatures, T_1 and T_2, is obtained from each of the above formula as follows :—

$$\text{Ln.}\frac{K_1}{K_2} = (T_1 - T_2)\left(\frac{A}{T_1 T_2} + B\right),$$

$$\text{Ln.}\frac{K_1}{K_2} = (T_1 - T_2)\frac{A}{T_1 T_2},$$

$$\text{Ln.}\frac{K_1}{K_2} = A\frac{T_1 - T_2}{T_1 T_2} + BLn\frac{T_1}{T_2}.$$

From each of the above equations, it is clearly seen that, when $(T_1 - T_2)$ is constant, i.e., the difference between these two temperatures is constant, the ratio of the velocity constants, K_1 and K_2, at these two different temperatures becomes smaller as these temperatures become higher.

In this case, when $T_1 = t$, and $T_2 = t + 10$, $K_{(t+10)}/K_t$ is commonly called as the "temperature coefficient" and the value of which is generally[45] $2-3$, according to the results obtained on the various chemical reactions.

In the case of the catalytic action by enzyme, the change of the temperature coefficient by rise of temperature is somewhat different from that in the case of the general chemical reactions, owing to the fact that, in this case, there accompanies the destruction of enzyme besides the increase of the reaction velecity by rise of temperature.

According to Tamman,[46] the temperature coefficient for emulsin is 7.14 between 60°–70°.

According to Bayliss,[4] the temperature coefficient for trypsin in 5.3 between 20°–30°.

According to H. Euler and Beth of Ugglas (1910),[47] the temperature coefficient of invertase is actually lower than that of inversion by acids, in fact, less than one half that of the latter; the same statement applies also to the action of lipase and of maltase when compared with the hydrolysis, by acid, of ethyl butyrate and of maltose respectively.

The result of the investigations which have been carried out on the temperature coeffieients in the case of the catalytic action by enzyme is as above stated. While, on papain, no detailed experimental result of this kind seems to have been obtained. Therefore, the author carried out the following experiment on this subject.

(A) Preliminary experiment

Casein-splitting action of papain reaches its equilibrium condition after a certain length of time of digestion and after that, its reaction does not go on further, no matter, how long the time of digestion may be taken. Therefore, in order to compare its reaction velocities at various temperatures, the length of time of digestion must be preferred so as not to reach its equilibrium condition in any reactions at those temperatures. For this purpose, the author found, at first, the optimum temperature for casein-splitting action of papain to be approximately at 80° in the conditions named and then, preferred the length of time of digestion for the present experiment not to reach the equilibrium condition by determining, in the following preliminary experiment, the reaction-velocity-curve at that optimum temperature.

Process of the preliminary experiment

In a series of 300 cc. Erlenmeyer's flasks, were placed 25 cc. of 2% casein solution, 5 cc. of 0.01% papain solution and water, making each total volume 150 cc. The mixture in each flask was allowed to

digest at 80°C in a constant temperature bath, during various lengths of time as in the Table (VI) (A). Further detailed process is just the same as in the case of the experiment (II).

Results of the preliminary experiment

Results obtained from the above experiment are given in the Table (VI) (A).

TABLE (VI) (A)

The quantities of casein *N* digested by papain during various lengths of time at the optimum temperature (80°C.).

No.	Time of digestion (min.)	c.c. of 2 % casein solution	c.c. of 0.01 % papain solution	c.c. of H_2O	c.c. of *N*/5 NaOH solution titrated(=*a*)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* undigested ($A=(20-a)2.5$)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* digested ($19.50-A$)
	Control.						
1	120+15	25	0	125	12.20	19.500	0
2	15	25	5	120	13.10	17.250	2.250
3	20+15	25	5	120	13.70	15.750	3.750
4	40+15	25	5	120	14.10	14.750	4.750
5	60+15	25	5	120	14.40	14.000	5.500
6	80+15	25	5	120	14.70	13.250	6.250
7	100+15	25	5	120	15.00	12.500	7.000
8	120+15	25	5	120	15.10	12.250	7.250

According to the result obtained, (1 hr.+15 min,) is preferred to be taken as the length of time of digestion for the present experiment.

(B) Main experiment

Experimental process

In a series of 300 cc. Erlenmeyer's flasks, were placed, 25 cc. of 2% casein solution, 5 cc. of 0.01% papain solution and water, making each total volume 150 cc.

The mixture in each flask was allowed to digest for (1 hr.+15 min.) in a constant temperature bath at various temperatures as in the Table (VI) (B) respectively. Further detailed process is just the same as in the case of the experiment (II).

Experimental results

Results obtained from the above experiment are given in the Table (VI) (B) and the temperature coefficients calculated are shown in the table (VII).

TABLE (VI) (B)

The quantities of casein N digested by papain at various temperatures of digestion.

No.	Temperature of digestion	c.c. of 2 % casein solution	c.c. of 0.01 % papain solution	c.c. of H_2O	c.c. of $N/5$ NaOH solution titrated ($=a$)	c.c. of $N/5$ NaOH solution corresponding to the total casein-N undigested ($A=(20-a)2.5$)	c.c. of $N/5$ NaOH solution corresponding to the total casein-N digested ($19.50-A$)
	Control.						
1	40°	25	0	125	12.20	19.500	0
2	20°	25	5	120	12.20	19.500	0
3	40°	25	5	120	12.30	19.250	0.250
4	50°	25	5	120	12.60	18.500	1.000
5	60°	25	5	120	13.35	16.625	2.875
6	70°	25	5	120	14.30	14.250	5.250
7	75°	25	5	120	14.80	13.000	6.500
8	80°	25	5	120	15.00	12.500	7.000
9	85°	25	5	120	14.80	13.000	6.500
10	90°	25	5	120	13.90	15.250	4.250
11	95°	25	5	120	13.40	16.500	3.000
12	Control. 95°	25	0	125	12.60	18.500	1.000

TABLES (VII)

Temperature coefficients at various ranges of temperature.

$K_{(t+10)}/K_t$.	Temperature coefficients
K_{50}/K_{40}	4.000
K_{60}/K_{50}	2.875
K_{70}/K_{60}	1.826
K_{80}/K_{70}	1.333
K_{90}/K_{80}	0.607

The results show that :—

(1) Casein-splitting action of papain is influenced very much by the temperature of digestion.

(2) Temperature coefficients at various ranges of temperture are calculated and shown in the Table (VII) and illustrated in a curve in Fig. (VII). Comparing those temperature coefficients :—

$$K_{50}/K_{40} > K_{60}/K_{50} > K_{70}/K_{60} > K_{80}/K_{70} > 1 > K_{90}/K_{80}.$$

i.e., the temperature coefficients in this case becomes smaller as the range of temperature becomes higher. This fact agrees with that shown by the equations which were proposed by the various authors.

(3) The optimum temperature in this case is 80°C. But, it is to be noticed that the action of papain is gradually retarded as the temperature of digestion rises. Consequently, the optimum temperature is a point at which the algebraic sum of the acceleration of reaction velocity by rise of temperature and the retardation of reaction velocity owing to the destruction of papain by rise of temperature expresses maximum intensity of casein decomposition and is not absolutely a point at which the temperature is optimum for papain itself.

Therefore, this optimum temperature should also vary somewhat by the lengths of time of digestion.

(VI) On the Influence of the Concentration of H-ions upon the Casein-sblitting Action of Papain

Enzymes, being generally colloids, are very sensitive to the action of electrolytes,[48] i. e., their action is generally accelerated in lower concentrations, and retarded in higher concentrations, of an electrolyte.

A remarkable case of this kind is that of H-ions, and on this subject many investigations have been made on various kinds of enzymes.[49]

Investigations which have been carried out on papain on this subject are as stated already in the introduction. But they do not sufficiently elucidate in detail the influence of the concentration of H-ions upon the casein-splitting action of papain, so it seems rather necessary to make a further study on this subject. Thus, the author has carried out the following experiment for this purpose. In order to obtain a definite H-ion concentration and keep it stable, buffer mixtures i. e., mixed solutions of acids, bases, and salts are commonly used. As the

constituents of these buffer mixtures might sometimes have a certain influence upon the action of enzyme, the author has adopted the following method of investigation.

At first, the influence of various concentrations of free HCl solution upon the casein-splitting action of papain was determined. Secondly, the influence of various concentrations of NaOH solution upon the same action was determined. And then, the influence of various concentrations of NaCl and KCl solutions upon the same actions was determined respectively, proving that neither Cl-ions nor Na-ions have any marked influence upon the same action in the range of concentrations tested H-ion concentrations of the solution at several important points were determined by the electrical measurements using Type K potentiometer.

(A) Influence of various concentrations of HCl solution

(a) In the case of using 25 cc. of 2% casein solution

Experimental process

Twenty five cc. of 2% casein solution were placed in a series of 300 cc. Erlenmeyer's flasks and to them were put, as in the Table (VIII) (Aa), various amounts of *N*/5 and *N*/50 HCl solution, various amounts of water and 5 cc. of 0.05% papain solution, making each total volume 150 cc. The mixture in each flask was allowed to digest at 40° in a constant temperature bath for (2 hr. + 15 min). Further detailed process is just the same as in the case of the experiment (II).

As precipitant, besides having added 5 cc. of *N*/1 HCl solution and 25 cc. of 20% Na_2SO_4 solution, a sufficient quantity of *N*/5 or *N*/50 NaOH solution was added in this case in order to neutralize the acidity of each digested solution.

Some mixtures in a series of Erlenmeyer's flasks gave turbidity before digestion, owing to the HCl solution added. Therefore, these conditions of turbidity were also noted in the table.

Experimental results

Results obtained from the above experiment are given in the Table (VIII) (Aa).

TABLE (VIII) (Aa)

The quantities of casein *N* digested by papain in various concentrations of HCl solution in the case of using 25 c.c. of 2% casein solution as substrate.

No.	c.c. of 2 % casein solution	c.c. of 0.05 % papain solution	c.c. of HCl solution	c.c. of H_2O	Condition of each mixed sample before digestion	c.c. of *N*/5 NaOH solution titrated (=*a*)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* undigested ($A=(20-a)2.5$)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* digested (19.000−*A*)
			N/5					
1	25	5	10	110		12.40	19.000	0
2	25	5	8	112		12.40	19.000	0
3	25	5	6	114		12.40	19.000	0
			N/50					
4	25	5	50	70		13.20	17.000	2.000
5	25	5	40	80	No turbidity.	13.95	15.125	3.875
6	25	5	30	90	Slightly turbid.	14.70	13.250	5.750
7	25	5	20	100	Most turbid, producing viscous white ppt.	16.70	8.250	10.750
8	25	5	18	102	Turbidity being increased.	17.60	6.000	13.000
9	25	5	16	104	No turbidity.	18.00	5.000	14.000
10	25	5	14	106		18.10	4.750	14.250
11	25	5	12	108		18.15	4.625	14.375
12	25	5	10	110		18.20	4.500	14.500
13	25	5	8	112		18.00	5.000	14.000
14	25	5	6	114		17.70	5.750	13.250
15	25	5	4	116		17.10	7.250	11.750
16	25	5	2	118		16.50	8.750	10.250
17	25	5	0	120		14.90	12.750	6.250
			N/5					
18	25	0	10	115		12.40	19.000	0
19	25	0	0	125		12.40	19.000	0

From the results stated above, we can see the following facts:

(1) Casein-splitting action of papain is accelerated by a definite range of HCl concentrations. An optimun HCl concentration is in the case of containing 10 cc. of *N*/50 HCl solution in the total volume 150 cc., *PH* of which being 6.5.

(2) As the HCl concentration increases more than this optimum concentration, casein-splitting action af papain is gradually retarded more markedly. Especially, in the HCl concentration which gives to the casein solution used as substrate maximum turbidity, i. e., in the case of containing 20 cc. of *N*/50 HCl solution in the total volume 150 cc. (*PH*=5.7), the retarding action of the acid increases suddenly at once. In the concentrations more than this the retarding action of HCl increases very slowly with increasing amount of the acid and finally casein-splitting action of papain stops. In this experiment, it is in the case of containing 6 cc. of *N*/5 HCl solution in the total volume of 150 cc.

(3) As seen above, the retardation of HCl solution upon the casein-splitting action of papain shows a tendency to increase suddenly at once in the concentration which gives to the casein solution maximum turbidity. This is probably due to the physico-chemical change of casein, along with the retarding action of HCl itself.

(b) In the case of using 35 cc. of 2% casein solution

Experimental process

Experimental process in this case is just the same as in the case of (a) except that 35 cc. of 2% casein solution in the total volume 150 cc. were used instead of 25 cc.

Some mixture in a series of Erlenmeyer's flasks gave also turbidity before digestion as in previous experiment, owing to the HCl solution added. Therefore these conditions of turbidity were also noted in the table.

Experimental results

Results obtained from the above experiment are given in the Table (VIII) (Ab).

TABLE (VIII) (Ab)

The quantities of casein *N* digested by papain in various concentrations of HCl solution in the case of using 35 c.c. of 2% casein solution as substrate.

No.	c.c. of 2 % casein solution	c.c. of 0.05 % papain solution	c.c. of HCl solution	c.c. of H_2O	Condition of each mixed sample before digestion	c.c. of *N*/5 NaOH solution titrated (=*a*)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* undigested ($A=(20-a)2.5$)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* digested ($27.250-A$)
			N/5					
1	35	5	16	94		8.90	27.750	0
2	35	5	14	96		8.90	27.750	0
3	35	5	12	98		8.90	27.750	0
4	35	5	10	100		9.15	27.125	0.625
5	35	5	8	102		9.50	26.250	1.500
6	35	5	6	104	No turbidity.	9.95	25.125	2.625
			N/50					
7	35	5	50	60	Slightly turbid.	11.25	21.875	5.875
8	35	5	40	70	Turbid.	11.90	20.250	7.500
9	35	5	30	80	Most turbid, producing viscous white ppt.	10.40	24.000	3.750
10	35	5	28	82	Turbidity	13.00	17.500	10.250
11	35	5	26	84	Turbidity being increased.	15.40	11.500	16.250
12	35	5	24	86	No turbidity.	16.00	10.000	17.750
13	35	5	22	88		16.50	8.750	19.000
14	35	5	20	90		16.90	7.750	20.000
15	35	5	18	92		17.00	7.500	20.250
16	35	5	16	94		17.10	7.250	20.500
17	35	5	14	96		17.20	7.000	20.750
18	35	5	12	98		17.10	7.250	20.500
19	35	5	10	100		16.90	7.750	20.000
20	35	5	8	102		16.50	8.750	19.000
21	35	5	6	104		16.10	9.750	18.000
22	35	5	4	106		15.30	11.750	16.000
23	35	5	2	108		14.40	14.000	13.750
24	35	5	0	110		12.95	17.625	10.125
			N/5					
25	35	0	10	105		8.90	27.750	0
26	35	0	0	115		8.90	27.750	0

The table shows clearly the following facts:

(1) Casein-splitting action of papain is accelerated by a definite range of HCl concentration, and in it we find an optimum ccncentration. These facts are just the same as in the case of (a), but in this case, the optimum HCl concentration is at a point in which 14 cc. of $N/50$ HCl solution were contained in the total volume 150 cc, while, in the case of (a), it was at a point in which 10 cc. of $N/50$ HCl solution were contained in the total volume 150 cc. Thus, it is clearly seen that the optimum HCl concentration increases as the concentration of casein increases. This is probably due to the fact that the quantity of H-ions absorbed by casein increases as the concentration of casein increases, as is the case in PH in the digested solutions proved similary ($PH=6,5$) in both cases.

(2) As the HCl concentration increases more than the optimum HCl concentration, casein-splitting action of papain is gradually retarded more markedly, This fact is also just the same as in the case (a), but, in this case, a sudden change of retardation caused by HCl in the concentration which gives maximum turbidity to the casein solution is observed more clearly, and we can see a very marked abnormal change given to the papain-activity. This is at a point in which 30 cc. of $N/50$ HCl solution were contained in the total volume 150 cc., containing also 10 cc. of $N/50$ HCl solution more than in the case of (a).

This phemomenon is also due to the fact that the quantity of H-ions adsorbed by casein increases as the concentration of casein increases, showing the same PH ($=5.7$) of the digested solutions in both cases.

(B) Influence of various concentrations of NaOH solution

Experimental process

Experimental process in this case is just the same as in the case of (A) except that various quantities of $N/5$ and $N/50$ NaOH solutions were used instead of $N/5$ and $N/50$ HCl solutions.

Experimental results.

Results obtained from the above experiment are given in the Table (VIII) (B).

TABLE (VIII) (B)

The quantities of casein *N* digested by papain in various concentrations of NaOH solution.

No.	c.c. of 2 % casein solution	c.c. of 0.05 % papain solution	c.c. of NaOH solution	c.c. of H_2O	c.c. of *N*/5 NaOH solution titrated(=*a*)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* undigested ($A=(20-a)2.5$)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* digested ($19.000-A$)
			N/50				
1a	25	5	50	70	13.20	17.000	2.000
2a	25	5	40	80	13.00	17.500	1.500
3a	25	5	30	90	12.80	18.000	1.000
4a	25	5	20	100	12.60	18.500	0.500
5a	25	5	10	110	12.40	19.000	0
6a	25	5	8	112	12.40	19.000	0
7a	25	5	6	114	12.40	19.000	0
			N/50				
8	25	5	50	70	12.45	18.875	0.125
9	25	5	40	80	12.55	18.625	0.375
10	25	5	30	90	12.65	18.375	0.625
11	25	5	20	100	12.90	17.750	1.250
12	25	5	18	102	12.95	17.625	1.375
13	25	5	16	104	13.00	17.500	1.500
14	25	5	14	106	13.10	17.250	1.750
15	25	5	12	108	13.20	17.000	2.000
16	25	5	10	110	13.55	16.125	2.875
17	25	5	8	112	13.90	15.250	3.750
18	25	5	6	114	14.30	14.250	4.750
19	25	5	4	116	14.95	12.625	6.375
20	25	5	2	118	15.65	10.875	8.125
21	25	5	0	120	16.60	8.500	10.500
22	25	0	0	125	12.40	19.000	0
			N/50				
1b	25	0	50	75	13.20	17.000	2.000
2b	25	0	40	85	13.00	17.500	1.500
3b	25	0	30	95	12.80	18.000	1.000
4b	25	0	20	105	12.60	18.500	0.500
5b	25	0	10	115	12.40	19.000	0
6b	25	0	8	117	12.40	19.000	0
7b	25	0	6	119	12.40	19.000	0

As seen in the table, casein-splitting action of papain is retarded by various concentrations of NaOH solution.

In the present experiment, we have arrived to the following conclusions :

(a) In the range of concentrations containing 0–20 cc. of *N*/50 NaOH solution in the total volume 150 cc., that retarding action gradually increases as the concentration of NaOH solution increases.

(b) In the range of concentrations containing 20–50 cc. of *N*/50 NaOH solution in the total volume 150 cc., that retarding action makes no perceptible increase.

(c) In the range of concentrations containing 6–10 cc. of *N*/5 NaOH solution in the total volume 150 cc., casein-splitting action of papain is entirely stopped.

(d) In the concentrations containing more than 10 cc. of *N*/5 NaOH solution in the total volume 150 cc., casein begins to be digested again and the intensity of that digestion increases as the concentration of NaOH solution increases. However, this is not due to the action of papain but due to the hydrolysis of casein by NaOH solution. This fact is shown quite clearly by the result in the table above stated.

(C) Influence of various cancentrations of NaCl and KCl solutions.

Experimental process

Experimental process in this case is just the same as in the case of (A) except that various amounts of *N*/5 and *N*/50 NaCl as well as KCl solutions were used instead of *N*/5 and *N*/50 HCl solutions.

Experimental results

Results obtained from the above experiment are given in the Table (VIII) (C).

In the range of the concentrations taken in the present experiment, casein-splitting action of papain receives no marked influence by the presence of NaCl or KCl. Consequently, in the experiments, (A) and (B), above stated, casein-splitting action of papain receives no influence by either Na-ions or Cl-ions, and the results obtained therefore, should be regarded, as expressing approximately the influence of H-ions as well as OH-ions upon the casein-splitting action of papain.

TABLE (VIII) (C)

The quantities of casein N digested by papain in various concentrations of NaCl as well as KCl solutions.

No.	c.c. of 2 % casein solution	c.c. of 00.5 % papain solution	c.c. of KCL or NaCL solution	c.c. of H_2O	c.c. of $N/5$ NaOH solution titrated(=a)	c.c. of $N/5$ NaOH solution corresponding to the total casein-N undigested ($A=(20-a)2.5$)	c.c. of $N/5$ NaOH solution corresponding to the total casein-N digested ($19.000-A$)
			$N/1$				
1	25	5	10	110	14.40	14.000	5.000
			$N/5$				
2	25	5	10	110	14.70	13.250	5.750
3	25	5	8	112	14.70	13.250	5.750
4	25	5	6	114	14.70	13.250	5.750
			$N/50$				
5	25	5	50	70	14.70	13.250	5.750
6	25	5	40	80	14.70	13.250	5.750
7	25	5	30	90	14.70	13.250	5.750
8	25	5	20	100	14.70	13.250	5.750
9	25	5	18	102	14.80	13.000	6.000
10	25	5	16	104	14.80	13.000	6.000
11	25	5	14	106	14.80	13.000	6.000
12	25	5	12	108	14.80	13.000	6.000
13	25	5	10	110	14.80	13.000	6.000
14	25	5	8	112	14.80	13.000	6.000
15	25	5	6	114	14.80	13.000	6.000
16	25	5	4	116	14.90	12.750	6.250
17	25	5	2	118	14.90	12.750	6.250
18	25	5	0	120	14.90	12.750	6.250
19	25	0	0	125	12.40	19.000	0
			$N/1$				
20	25	0	10	115	12.40	19.000	0

(VII) On the Question of the Formation of Amino acids by Papain in its Casein-splitting Action

On this question, many investigations were carried out by various authors, but the results obtained, did not, on the whole, agree with one another. Some authors, obtained a perceptible quantity of amino acids in the digestion products of proteins of papain, while others, did not perceive any quantity of amino acids in their experiments. (See

the introduction.) But, according to a thorough observation of all the literature stated above, it seems as though papain forms mainly albumoses and peptones in its degestion products of proteins, and its ability of forming amino acids is very weak and varies somewhat by (a) kinds of proteins, (b) lengths of digestion time, (c) heights of digestion temperature and (d) by the presence or absence of HCN or other antiseptics, etc. As the elucidation of this question is one of the very important problems in the physico-chemical investigation of casein-splitting action of papain, the following experiments have been carried out on this subject as follows:

Experimental process

In a series of 300 cc. Erlenmeyer's flasks were placed, as in the Table (IX), definite quantities of 2% casein solution, 0.05% papain solution, ($N/5$ KCN+$N/5$ HCl) neutral solution and $N/50$ HCl solution, making each total volume 150 cc.

The mixture in each flask was digested for (2 hr.+15 min.) in a constant temperature bath at 40°C and 80°C respectively. Casein undigested in each flask was precipitated by the same method as before, and then, filtered and washed with 5% Na_2SO_4 solution. The filtrate obtained from each flask including washing was introduced into a 250 cc. measuring flask and made total volume 250 cc. with water. 100 cc. of the filtrate were taken from each flask as sample and the following two quantitative determinations were carried out:

(a) The quantity of amino N in 100 cc. of each filtrate was determined by the Sörensen's method and cc. of $N/5$ NaOH solution corresponding to the quantity of amino N in the whole cc. of the filtrate was then calculated.

(b) A hundred cc. of each filtrate was hydrolysed completely in Erlenmeyer's flask by adding 120 cc. of conc. hydrochloric acid (38%) and boiling for 10 hours on the sand bath with reflux condenser. The content in each flask was, then, filtered and the residue washed thoroughly with water. The filtrate and the washing were introduced into a 250 cc. measuring flask and made the total volume 250 cc. with water. A hundred cc. of this solution was taken as sample and the quantity of amino N was determined by the Sörensen's method. Amount of $N/5$ NaOH solution corresponding to the quantity of amino N produced when the whole nitrogenous substances in the filtrate was hydrolysed, was then calculated.

Experimental results

Results obtained from the above experiment are given in the Table (IX).

TABLE (IX)

The quantities of amino acids *N* formed during the digestion by papain.

No.	c.c. of 2 % casein solution	c.c. of 0.05% papain solution	c.c. of KCN solution	c.c. of HCL solution	c.c. of HCL solution	c.c. of H_2O	Temperature of digestion	c.c. of *N*/5 NaOH solution corresponding to the amino *N* formed in the filtrate degested by papain	c.c. of *N*/5 NaOH solution corresponding to the total amino *N* formed by the total hydrolysis of the digested filtrate
1	25	0	0	0	0	125	40	0	0
2	0	20	0	0	0	130	40	0	0
3	25	20	0	0	0	105	40	0.75	10
4	25	20	*N*/50 10	*N*/50 10	0	85	40	0.75	10
5	25	20	*N*/50 10	*N*/50 10	*N*/50 10	75	40	0.75	10
6	25	0	0	0	0	125	80	0	0
7	0	20	0	0	0	130	80	0	0
8	25	20	0	0	0	105	80	0.75	10
9	25	20	*N*/50 10	*N*/50 10	0	85	80	0.75	10
10	25	20	*N*/50 10	*N*/50 10	*N*/50 10	75	80	0.75	10

This experiment has been carried out under the several combinations of most favorable conditions upon the casein-splitting action of papain, i.e.,

(a) in the presence of a certain quantity of HCN which gives a marked acceleration as will be stated in the following chapter.

(b) in the presence of quantities of HCN and H-ions which give a marked acceleration,

(c) under the combination of the conditions above stated and optimum temperature.

But, under whatever favorable conditions it may be, the quantity of amino N formed by papain in its casein-splitting action is very little, as seen in the table. When this quantity of amino N is compared with that of the total amino N obtained by the total hydrolysis of nitrogenous substances in the digested filtrate at the concentration of 20% HCl, the ratio was found to be 0.75/10.

Therefore, papain forms mainly polypeptides such as albumoses and peptones in its casein-splitting action and its ability of forming amino acids should be regarded as either very little or entirely negative.

Summary on the Fundamental Properties :—

Papain was prepared from the milky latex of the green fruits of papaya trees produced at Heitō, Formosa and purified by the method previously described. The fundamental properties of the casein-splitting action of papain were studied with this preparation. Results obtained are given in tables. Therefore, only important points are summarized as follows :

(1) The quantity of casein digested by papain during a unit time is directly proportional to the concentration of papain, in the range of the reaction which does not draw near its equilibrium condition. Schütz's law cannot be applied in this case.

(2) The curvature of the reaction-velocity-curves of the casein-splitting action of papain makes nearly straight lines, in the range of the reactions which do not approach their equilibrium conditions. Therefore, the law of mass action is not strictly applicable and the value K corresponding to the constant in the mono-molecular reaction increases gradually as the reaction proceeds.

(3) Comparing 3 velocity curves in the cases of using 3 various concentrations of casein as substrate from the beginning of each reaction, these curves were observed to be very near with one another, in the range of the reactions which do not draw near their equilibrium conditions. This shows the fact that the law of mass action is not strictly applicable also in this case as in the case (2),

(4) Comparing temperature-coefficients at various ranges of temperature on the casein-splitting action of papain, the following order is obtained :—

$$\frac{K_{50}}{K_{40}} > \frac{K_{60}}{K_{50}} > \frac{K_{70}}{K_{60}} > \frac{K_{80}}{K_{70}} > 1 > \frac{K_{90}}{K_{80}}$$

i.e., the temperature coefficients in this case becomes smaller as the range of temperature becomes higher. This fact agrees with that shown by the equations which were proposed by Van't Hoff, Arrhenius, and D.M. Kooy, respectively.

(5) The optimum temperature for the casein-splitting action of papain was fouud to be 80°C. But it is to be noticed that this optimum temperature may also vary somewhat by the lengths of time of digestion.

(6) The optimum hydrogen ion concentration for the casein-splitting action of papain was found to be at the point of $PH=6.5$.

(7) The PH-activity in the casein-splitting action of papain receives, at one time, a sudden abnormal change at the point of $PH=5.7$.

This is a point at which casein was precipitated producing maximum turbidity. Therefore, this abnormal change should be regarded as due to the physico-chemical change of casein caused by hydrogen ions.

(8) Casein-splitting action of papain is retarded by various concentrations of NaOH solution and its action is entirely stopped at the concentrations of $N/125 \sim N/75$ NaOH.

(9) Under whatever favorable conditions it may be, the quantity of amino N formed by papain in its casein-splitting action is very little.

When this quantity of amino N is compared with that of the total amino N contained by the total hydrolysis of nitrogenous matters in the digested filtrate at the concentration of 20% HCl, the ratio is found to be 0.75/10.

Therefore, papain forms mainly polypeptides such as albumoses and peptones in its casein-splitting action and its ability of forming amino acids should be regarded as either very small or entirely negative.

(C) On the Mechanism of the Acceleration of HCN solution upon the Casein-splitting Action of Papain

Investigations which have been carried out on the acceleration of HCN solution upon the proteolytic action of papain are as already stated in the Part (a) of the introduction.

From these investigations, no scfficient results can be seen on the mechanism of the acceleration of HCN solution upon the proteolytic action of papain. Therefore, in ordor to give a further clear explanation on its mechanism the auther carried out the following experiments on this subject.

(I) On the influence of the (KCN+HCl) neutral solution upon the casein-splitting action of papain with special reference to the concentration and the time of digestion

According to Mendel and Blood,[50] the marked acceleration of HCN solution upon the proteolytic action of papain can not be attributed to the favorable concentrations of H-ions. This conclusion was obtained from the following two experimental data.

(1) Preparing three solutions equivalent in the concentration of H-ions with hydrogen sulphide, boric acid, and HCN, which have practically the same degree of electrical dissociation with one another, they observed the influence of these three acid solutions upon the egg-white-splitting action as well as upon Witte's peptone-splitting action of papain and obtained the result that hydrogen sulphide resembles HCN in its action of accelerating proteolysis, but not the boric acid.

(2) In the experiments in which the effect of HCN was determined over a considerable range of acidity and alkalinity, HCN is effective even when considerable amounts of hydrochloric acid or sodium carbonate are present, whereas, in no concentration did either this acid or alkali alone call forth a marked digestion of "peptone" or of egg-white.

Observing these two data, that conclusion obtained by them seems to be right. In order to examine this conclusion more definitely, the author has carried out the following experiments on this subject.

Experimental process in the case of concentration test.

In a series of 300 cc. Erlenmeyer's flasks, were placed, 25 cc. of 2% casein solution and to them were put as in the Table (X) (A), various amounts of ($N/5$ or $N/50$ KCN+$N/5$ or $N/50$ HCl) neutral solution, 5 cc. of 0.05% papain solution and various amounts of water, making each total volume 150 cc. The mixture in each flask was allowed to digest for (2 hr.+15 min.) in a constant temperature bath at 40°C. The further method of this experiment was similar to the experiment (II) of part (B).

Experimental process in the case of the digestion time test.

In a series of 300 cc. Erlenmeyer's flasks, were placed, 25 cc. of 2% casein solution, [(2 cc. of $N/50$ KCN+2 cc. of $N/50$ HCl) or (10 cc. of $N/50$ KCN+10 cc. of $N/50$ HCl)] neutral solution, 5 cc. of 0.05% papain solution and water, making each total volume 150 cc. The mixture in each flask was allowed to digest in a constant temperature bath at 40°C during various lengths of time as in the Table (X) (B). Further detailed process is just the same as in the experiment (II) of part (B).

In this experiment the following two tests were also made and the results obtained were compared with those of the above experiment:—

(a) In the case of the sbsence of (KCN+HCl) neutral solution in the mixture above stated.

(b) In the case of the presence of 1 cc. of toluene instead of using (KCN+HCl) neutral solution in the mixture above stated.

Experimental results

Results obtained from the above experiment are given in the Table (X) (A) and Table (X) (B).

TABLE (X) (A)

The quantities of casein *N* digested by papain in various concentration of (KCN+HCl) neutral solution.

No.	c.c. of 2 % casein solution	c.c. of 0.05 % papain solution	c.c. of KCN solution	c.c. of HCL solution	c.c. of H_2O	c.c. of *N*/5 NaOH solution titrated (=*a*)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* undigested (*A*=(20−*a*)2.5)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* digested (19.750−*A*)
			N/1	*N*/1				
1	25	5	10	10	100	19.30	1.750	18.000
			N/5	*N*/5				
2	25	5	10	10	100	19.30	1.750	18.000
3	25	5	8	8	104	19.30	1.750	18.000
4	25	5	6	6	108	19.20	2.000	17.750
			N/50	*N*/50				
5	25	5	50	50	20	19.20	2.000	17.750
6	25	5	40	40	40	19.10	2.250	17.500
7	25	5	30	30	60	19.10	2.250	17.500
8	25	5	20	20	80	19.00	2.500	17.250
9	25	5	18	18	84	19.00	2.500	17.250
10	25	5	16	16	88	19.00	2.500	17.250
11	25	5	14	14	92	18.90	2.750	17.000
12	25	5	12	12	96	18.80	3.000	16.750
13	25	5	10	10	100	18.70	3.250	16.500
14	25	5	8	8	104	18.70	3.250	16.500
15	25	5	6	6	108	18.60	3.500	16.250
16	25	5	4	4	112	18.50	3.750	16.000
17	25	5	2	2	116	18.40	4.000	15.750
18	25	5	0	0	120	16.70	8.250	11.500
			N/1	*N*/1				
19	25	0	10	10	105	12.10	19.750	0
20	25	0	0	0	125	12.10	19.750	0

TABLE (X) (B)

The quantities of casein *N* digested by papain during various lengths of time,

(a) in the presence of (KCN+HCL) neutral solution,
(b) in the presence of toluene,
(c) in the presence of no antiseptic.

No.	Time of digestion (min.)	c.c. of 2 % casein solution	c.c. of 0.05% papain solution	c.c. of KCN solution or Toluol	c.c. of HCL solution	c.c. of H_2O	c.c. of *N*/5 NaOH solution titrated (=*a*)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* undigested ($A=(20-a)2.5$)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* digested (19.750 − *A*)
				N/5	*N*/5				
1a	15	25	5	10	10	100	16.90	7.750	12.000
2a	60+15	25	5	10	10	100	19.20	2.000	17.750
3a	120+15	25	5	10	10	100	19.40	1.500	18.250
4a	180+15	25	5	10	10	100	19.60	1.000	18.750
5a	300+15	25	5	10	10	100	19.65	0.875	18.875
				N/50	*N*/50				
1b	15	25	5	2	2	116	14.20	14.500	5.250
2b	60+15	25	5	2	2	116	17.70	5.750	14.000
3b	120+15	25	5	2	2	116	18.60	3.500	16.250
4b	180+15	25	5	2	2	116	18.90	2.750	17.000
5b	300+15	25	5	2	2	116	19.20	2.000	17.750
1c	15	25	5	0	0	120	12.80	18.000	1.750
2c	60+15	25	5	0	0	120	14.70	13.250	6.500
3c	120+15	25	5	0	0	120	16.60	8.500	11.250
4c	180+15	25	5	0	0	120	17.50	6.250	13.500
5c	300+15	25	5	0	0	120	18.20	4.500	15.250
				Toluol					
1d	15	25	5	1	0	119	12.40	19.000	0.750
2d	60+15	25	5	1	0	119	13.40	16.500	3.250
3d	120+15	25	5	1	0	119	14.40	14.000	5.750
4d	180+15	25	5	1	0	119	15.10	12.250	7.500
5d	300+15	25	5	1	0	119	15.80	10.500	9.250
1e	0	25	0	0	0	125	12.10	19.750	0
				N/5	*N*/5				
2e	300+15	25	0	10	10	105	12.10	19.750	0

As seen in the table, casein-splitting action of papain is accelerated very markedly by addition of (KCN+HCl) neutral solution in a definite range of concentration.

According to the previous experimental results, KCl gives no marked acceleration on this kind of papain action. Therefore, this accelerating action of (KCN+HCl) neutral solution should be regarded as due to the HCN in the solution. Furthermore, this HCN solution being made neutral in this case, it is very clear that this accelerating action is not due to the H-ions in the concentrations favorable on the action of papain.

(II) On the influence of KCN solution upon the casein-splitting action of papain

Mendel and Blood[51] were not sure whether the acceleration of HCN solution upon the proteolytic action of papain was due to the CN-radical or not, and carried out an experiment on the influence of some CN-compounds related to HCN upon the egg-white-splitting action as well as upon Witt's peptone-splitting action of papain with a comparison with that of HCN.

Observing their results, a further detailed experiment was carried out on the influence of KCN solution upon the casein-splitting action of papain, as the author thought it very important for his present investigation to elucidate the mechanism of the acceleration of HCN solution.

Experimental process

In a series of 300 cc. Erlenmeyer's flasks, were placed, 25 cc. of 2% casein solution and into them, were put, as in the Table (XI), various amounts of *N*/5 or *N*/50 KCN solution, 5 cc. of 0.05% papain solution and various amounts of water, making each total volume 150 cc.

The mixture in each flask was allowed to digest for (2 hr.+15 min.) in a constant temperature bath at 40°C. The further method used in this experiment was similar to the experiment (II) of part (B). As precipitant, besides having added 5 cc. of *N*/1 HCl solution and 25 cc. of 20% Na_2SO_4 solution, a sufficient quantity of *N*/5 or *N*/50 HCl solution was added in order to neutralize the alkalinity of each digested solution.

Experimental results

Result obtained from the above experiment is given in the Table (XI).

TABLE (XI)

The quantities of casein *N* digested by papain in various concentrations of KCN solution.

No.	c.c. of 2 % casein solution	c.c. of 0.05 % papain solution	c.c. of KCN solution	c.c. of HCL solution	c.c. of H_2O	c.c. of *N*/5 NaOH solution titrated (=*a*)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* undigested (*A*=(20−*a*)2.5)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* digested (19.750−*A*)
			N/1					
1	25	5	10	0	110	17.50	6.250	13.500
			N/5					
2	25	5	10	0	110	18.60	3.500	16.250
3	25	5	8	0	112	18.70	3.250	16.500
4	25	5	6	0	114	18.80	3.000	16.750
			N/50					
5	25	5	50	0	70	18.90	2.750	17.000
6	25	5	40	0	80	19.00	2.500	17.250
7	25	5	30	0	90	19.00	2.500	17.250
8	25	5	20	0	100	19.00	2.500	17.250
9	25	5	18	0	102	19.00	2.500	17.250
10	25	5	16	0	104	19.00	2.500	17.250
11	25	5	14	0	106	18.90	2.750	17.000
12	25	5	12	0	108	18.80	3.000	16.750
13	25	5	10	0	110	18.70	3.250	16.500
14	25	5	8	0	112	18.70	3.250	16.500
15	25	5	6	0	114	18.60	3.500	16.250
16	25	5	4	0	116	18.50	3.750	16.000
17	25	5	2	0	118	18.40	4.000	15.750
18	25	5	0	0	120	16.70	8.250	11.500
			N/1					
19	25	0	10	0	115	12.10	19.750	0
20	25	0	0	0	125	12.10	19.750	0

The pesults reached are as follows :

(1) Casein-splitting action of papain is accelerated very markedly by a definite range of concentrations of KCN solution and among which, there is an optimum zone of concentrations of KCN solution. In this experiment, that optimum zone is in the case of containing 16–40 cc. of $N/50$ KCN solution in the total volume 150 cc.

(2) As KCN is a salt consisting of a very weak acid HCN and a very strong base KOH, it is hydrolyzed in its water solution into HCN+KOH.

Consequently the accelerating action of KCN solution should be regarded as due to the HCN+KOH solution. According to the previous experimental result, Table (VIII) (B), KOH solution not, only gives no acceleration but rather gives a marked retardation.

Therefore, the accelerating action in this case should be regarded as due to the HCN.

As above stated, HCN, being an acid which is very weak in its dissociation degree, the author thinks that the accelerating action in this case should be regarded as due to the molecules of HCN, but not to the CN-ions.

(3) When the accelerating action (a) of (KCN+HCl) neutral solution and that (b) of KCN solution upon the casein-splitting action of papain are compared, it seems as though in a range of the concentrations containing 0–20 cc. of $N/50$ KCN solution in the total volume 150 cc., the intensity of the accelerating action is just the same in both cases. This is probably due to the following fact :—

In the case (b), KCN being hydrolyzed in its water solution, its solution becomes alkaline. But, in such an alkalinity caused by such a range of KCN concentrations, the retardation due to that alkalinity can't be observed, because of the antagonistic action due to the acceleration of HCN.

In both cases, the accelerating action upon the casein-splitting action of papain gradually increases as the concentration of KCN increases. But, the range of KCN concentrations increasing the intensity of that acceleration is wider in the case (a) than in the case (b).

This is probably due to the following fact :—

In the case (b), the alkalinity produced by hydrolysis of the salt is not neutralized, as in the case of (a), where its intensity is gradually increased as the concentration of KCN increases. Concequently, the

ntensity of the retardation due to that alkalinity becomes more marked as the salt concentrations increase, and finally is observed to overbalance the antagonistic action due to the acceleration of HCN.

(III) On the influence of HCN solution upon the casein-splitting action of papain, in the various concentrations of H-ions

In the previous experiments, the author verified that the marked accelerating influence of HCN solution upon the casein-splitting action of papain was not either due to the favorable concentration of H-ions or to CN-ions.

Then, the author, assumed that the marked acceleration of HCN solution is mainly due to the undissociated molecules of HCN.

If this assumption is true, the accelerating action of HCN should be raised by the addition of HCl in a definite range of the concentrations. This is thought from the fact that the acid HCl, which is very strong in its dissociation degree, being added to the acid HCN, very weak in its dissociation, the degree of dissociation of the latter must be decreased, forming much more undissociated molecules of HCN. Furthermore, in comparing the accelerating action of (a) (KCN+HCl) a neutral solution and that of (b) KCN solution upon the casein-splitting action of papain, the author observed that, in a range of the concentrations containing 0–20 cc. of *N*/50 KCN solution in the total volume 150 cc., the intensity of the accelerating action of KCN is just the same in both cases. And, for that reason, the author infers that, in such an alkalinity which is cased by such a range of KCN concentrations, the retardation due to that alkalinity in the case of (b) can't be observed because of the antagonistic action due to the acceleration of HCN.

In order to give a verification whether these assumptions are true or not, the author has carried out the following experiment.

Experimental process :—

In a series of 300 cc. Erlenmeyer's flasks, were placed, 25 cc. of 2% casein solution and into them, were put, as in the Tables, (XII) (A), (XII) (B), 10 cc. of *N*/5 KCN solution and 10 cc. of *N*/5 HCl solution, making the mixture in each flask neutral, and then were added various amounts of *N*/1 and *N*/50 HCl or *N*/1 and *N*/50 NaOH solutions, 5 cc. of 0.5% papain solution and various amounts of water, making each total volume 150 cc.

The mixture in each flask was allowed to digest for (2 hr. + 15 min.) in a constant temperature bath at 40°C. Further detailed process is just the same as in the experiment (VI) of part (B).

As a comparative test the following experiment was carried out:—

In a series of 300 cc. Erlenmeyer's flasks, were placed, 25 cc. of 2% casein solution and into them, were put, as in the Tables, (XII) (A), (XII) (B), various amounts of *N*/1 and *N*/50 HCl or *N*/1 and *N*/50 NaOH solutions, 5 cc. of 0.05% papain solution and various amounts of water, making each total volume 150 cc.

The mixture in each flask was treated by the same process as above.

Experimental results

Results obtained from the above experiments are given in the Tables, (XII) (A), (XII) (B).

As seen in the tables, casein-splitting action of papain is also accelerated very markedly by HCN, even in a fairly wide range of concentrations of H-ions. This result verifies the assumption that the marked acceleration of HCN solution is mainly due to the undissociated molecules of HCN.

Casein-splitting action of papain is however, retarded by various concentrations of NaOH solution. According to the above experimental result, that retarding actoin gradually increases, in the range of concentrations containing 0–20 cc. of *N*/50 NaOH solution in the total volume 150 cc. But, when (10 cc. of *N*/5 KCN + 10 cc. of *N*/5 HCl) neutral solution is added furthermore to the mixture in each flask before digestion, the acceleration of HCN produced by that neutral solution added, is observed very markedly, and the intensity of that acceleration in each mixture becomes entirely equal with one another in such an alkalinity as above stated.

In this case, the retardation due to the alkaline solution can't be observed, receiving antagonistic action due to the acceleration of HCN. This fact also verifies the assumption which the author has shown before.

(IV) On the influence of the reversibility in the combination of papain and HCN upon the casein-splitting action of papain

Mendel and Blood[52] carried out an experiment, on the influence of the reversibility in the combination of papain and HCN upon the egg-white-splitting action of papain.

TABLE (XII) (A)

The quantities of casein *N* digested by papain, in the presence of HCN solution, in various concentrations of HCL solution.

No.	c.c. of 2 % casein solution	c.c. of 0.05% papain solution	c.c. of HCL solution	c.c. of KCN solution	c.c. of HCL solution	c.c. of H_2O	c.c. of *N*/5 NaOH solution titrated (=*a*)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* undigested ($A=(20-a)2.5$)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* digested ($27.750-A$)
			N/5	*N*/5	*N*/5				
1a	35	5	12	10	10	78	8.90	27.750	0
2a	35	5	6	10	10	84	10.95	22.625	5.125
3a	35	5	5	10	10	85	14.45	13.875	13.875
4a	35	5	4	10	10	86	18.40	4.000	23.750
			N/50						
5a	35	5	30	10	10	60	19.40	1.500	26.250
6a	35	5	28	10	10	62	19.40	1.500	26.250
7a	35	5	26	10	10	64	19.40	1.500	26.250
8a	35	5	24	10	10	66	19.50	1.250	26.500
9a	35	5	20	10	10	70	19.50	1.250	26.500
10a	35	5	14	10	10	76	19.50	1.250	26.500
11a	35	5	6	10	10	84	19.50	1.250	26.500
12a	35	5	2	10	10	88	19.50	1.250	26.500
13a	35	5	0	0	0	110	12.95	17.625	10.125
			N/5						
1b	35	5	12	0	0	98	8.90	27.750	0
2b	35	5	6	0	0	104	9.95	25.125	2.625
3b	35	5	5	0	0	105	11.25	21.875	5.875
4b	35	5	4	0	0	106	11.90	20.250	7.500
			N/50						
5b	35	5	30	0	0	80	10.40	24.000	3.750
6b	35	5	28	0	0	82	13.00	17.500	10.250
7b	35	5	26	0	0	84	15.40	11.500	16.250
8b	35	5	24	0	0	86	16.00	10.000	17.750
9b	35	5	20	0	0	90	16.90	7.750	20.000
10b	35	5	14	0	0	96	17.20	7.000	20.750
11b	35	5	6	0	0	104	16.10	9.750	18.000
12b	35	5	2	0	0	108	14.40	14.000	13.750
13b	35	5	0	0	0	110	12.95	17.625	10.125
1c	35	0	0	0	0	115	8.90	27.750	0

TABLE (XII) (B)

The quantities of casein *N* digested by papain, in the presence of HCN solution, in various concentrations of NaOH solution.

No.	c.c. of 2 % casein solution	c.c. of 0.05% papain solution	c.c. of NaOH solution	c.c. of KCN solution	c.c. of HCL solution	c.c. of H_2O	c.c. of *N*/5 NaOH solution titrated (=*a*)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* undigested (*A*=(20−*a*)2.5)	c.c. of *N*/5 NaOH solution corresponding to the total casein-*N* digested (19.000−*A*)
1a	25	5	*N*/1 10	*N*/5 10	*N*/5 10	90	13.20	17.000	2.000
2a	25	5	6	10	10	94	13.10	17.250	1.750
3a	25	5	2	10	10	98	18.20	4.500	14.500
4a	25	5	*N*/50 20	10	10	80	19.30	1.750	17.250
5a	25	5	12	10	10	88	19.30	1.750	17.250
6a	25	5	8	10	10	92	19.30	1.750	17.250
7a	25	5	6	10	10	94	19.30	1.750	17.250
8a	25	5	4	10	10	96	19.30	1.750	17.250
9a	25	5	2	10	10	98	19.30	1.750	17.250
10a	25	5	0	0	0	120	16.60	8.500	10.500
1b	25	5	*N*/1 10	0	0	110	13.20	17.000	2.000
2b	25	5	6	0	0	114	12.80	18.000	1.000
3b	25	5	2	0	0	118	12.40	19.000	0
4b	25	5	*N*/50 20	0	0	100	12.90	17.750	1.250
5b	25	5	12	0	0	108	13.20	17.000	2.000
6b	25	5	8	0	0	112	13.90	15.250	3.750
7b	25	5	6	0	0	114	14.30	14.250	4.750
8b	25	5	4	0	0	116	14.95	12.625	6.375
9b	25	5	2	0	0	118	15.65	10.875	8.125
10b	25	5	0	0	0	120	16.60	8.500	10.500
1c	25	0	0	0	0	125	12.40	19.000	0
2c	25	0	*N*/1 10	*N*/5 10	*N*/5 10	95	13.20	17.000	2.000

In that experiment, they examined the intensity of the egg-white-splitting action of papain, in the following three cases :—

(a) In the case when papain was treated with HCN before digestion process and was left to stand for several hours.

(b) In the case when papain was treated with HCN previously and afterwards; HCN was removed from a portion of the mixture by bubbling air through it for three hours.

(c) In the case when papain was treated with HCN previously and after that, HCN was removed by dialysis for 18 hours against running water with toluene as an antiseptic.

According to that result, a marked acceleration was observed in the case (a), but, no acceleration was observed in the cases, (b) and (c). Accordingly, in this case, the reversibility in the combination of papain and HCN gave also a reversible influence upon the egg-white-splitting action of papain. From this point of view, the author carried out the following experiment on the influence of the reversibility in the combination of papain and HCN upon the casein-splitting action of papain.

Experimental process

In a series of 300 cc. Erlenmeyer's flasks, were placed, 5 cc. of 0.05% papain solution, 4 cc. of neutral solution (2 cc. of N/50 KCN solution + 2 cc. of *N*/50 HCl solution) and 66 cc. of water. From the mixture in each flask, HCN was removed by bubbling CO_2 free air through it for 5 hours. And then, the glass tube which was inserted into each mixture and through which CO_2 free air was passed, was washed thoroughly with 50 cc. of water, adding the washing into each mixture and into it, were put, 25 cc. of 2% casein solution, making each total volume 150 cc.

The mixture in each flask was allowed to digest for (2 hr. + 15 min.) in a constant temperature bath at 40°C.

As a comparative experiment, a series of mixtures was prepared as in the Table (XIII) and the mixture in each flask was allowed to digest by the same method as above.

Further detailed process is just the same as in the experiment (II) of part (B).

Experimental results

Results obtained from the above experiment are given in the Table (XIII).

TABLE (XIII)

The influence of the reversibility in the combination of papain and HCN upon the casein-splitting action of papain.

No.	c.c. of 0.05% papain solution	c.c. of KCN solution	c.c. of HCL solution	c.c. of H_2O	time required for passing through CO_2-free air (hr.)	c.c. of 2 % casein solution	c.c. of $N/5$ NaOH solution titrated ($=a$)	c.c. of $N/5$ NaOH solution corresponding to the total casein-N undigested ($A=(20-a)2.5$)	c.c. of $N/5$ NaOH solution corresponding to the total casein-N digested ($19.730-A$)
		$N/50$	$N/50$						
1	5	2	2	116	(5)	25	14.70	13.250	6.500
2	5	2	2	116	(5)	25	14.70	13.250	6.500
3	5	2	2	116	0	25	17.70	5.750	14.000
4	5	2	2	116	0	25	17.70	5.750	14.000
5	5	0	0	120	(5)	25	14.70	13.250	6.500
6	5	0	0	120	0	25	14.70	13.250	6.500
7	0	0	0	125	0	25	12.10	19.750	0

From the results, it will be clearly seen that the reversibility in the combination of papain and HCN gives a reversible influence upon the casein-splitting action of papain. This result coincides entirely with that obtained by Mendel and Blood.

(V) Conclusions

On the mechanism of the acceleration of HCN solution upon the casein-splitting action of papain, the following conclusions are drawn from the experiments above stated.

(A) The marked acceleration of HCN solution upon the casein-splitting action of papain should be regarded as mainly due to the undissociated molecules of HCN.

(B) The reversibility in the combination of papain and HCN gives a reversible influence upon the casein-splitting action of papain.

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