

STUDIES ON ENZYME ACTION.

XV. FACTORS INFLUENCING THE PROTEOLYTIC ACTIVITY OF PAPAIN.

By EDWARD M. FRANKEL.

(From the Harriman Research Laboratory, The Roosevelt Hospital,
New York.)

(Received for publication, May 25, 1917.)

The plant protease, papain, has been the subject of numerous studies. The earlier literature on the subject has been reviewed in some detail by Mendel and Blood (1910), whose paper embodies the results of one of the few careful investigations of this ferment. The work of these authors is, however, almost entirely qualitative in character. In extending the general plan of enzyme study that has been undertaken in this laboratory, it seemed advisable to reinvestigate some of the facts concerning this enzyme, employing some of the more accurate methods that have been recently developed. This ferment lends itself very well to chemical study since it may be obtained in large amounts of fairly uniform activity.

In this paper some observations concerning the purification of the active material and a consideration of the influence of acidity and the quantitative relationship between enzyme and substrate will be presented. The peculiar behavior of HCN in accelerating the action of papain has been studied further.

Purification of Papain.

In view of the method by which commercial papain is produced,¹ it seemed desirable that a more refined material be used in this work, especially since some of the contaminating material might have a deleterious action on the enzyme. It also might be

¹ Pratt, D. S., *Philippine J. Sc.*, 1915, x, 1.

expected that a refined material would be more uniform in composition and would therefore make the different experiments more comparable.

The papain as obtained from Parke, Davis and Company was a light brown finely divided powder nearly all of which was soluble in water, giving a yellow to brown solution. The insoluble material settled rapidly and the solution could readily be removed from it by decantation. A few preliminary experiments were carried out with a view to determining to what extent purification of the active material could be accomplished.

15 gm. of papain were ground up with 500 cc. of distilled water and allowed to stand over night. The next morning a portion of the suspension was filtered through asbestos and treated as follows.

A. 50 cc. of the filtered solution were treated with 150 cc. of acetone and the mixture was allowed to stand 2 hours. The precipitate that formed was centrifuged off and the mother liquor decanted. The precipitate was then taken up in 100 cc. of water. This is referred to as Solution A.

B. 50 cc. of the filtered solution were added to 100 cc. of acetone and then treated as in A, the water solution of the resulting precipitate being Solution B.

C. 50 cc. of the filtered solution were added to 250 cc. of 95 per cent alcohol and then treated as in A, the water solution of the resulting precipitate being Solution C.

As controls, the original filtered and unfiltered solutions diluted with an equal volume of water were used. These solutions were D and E respectively.

To test the activity of the solutions 5 cc. were allowed to act on 25 cc. portions of 1 per cent gelatin containing 0.2 per cent of tricresol as a preservative. The solutions were incubated at 37° for 17 hours and the increase in "formol" titration over the blanks was used as a measure of the activity.

Solution.	Formol titration.			
	Substrate blank.	Ferment blank.	Ferment + substrate.	Action.
A	2.65	0.35	4.00	1.00
B	2.65	0.30	4.00	1.05
C	2.65	0.45	4.90	1.80
D	2.65	1.00	4.60	0.95
E	2.65	1.10	5.00	1.25

From the above experiment it is apparent that the active material may be concentrated by the precipitation with two to three volumes of acetone or with five volumes of 95 per cent alcohol; the latter procedure in addition to effecting a concentration of the active material seems to remove some of the inhibiting contaminants. It is also to be noted that the precipitation removes from the active material a relatively large amount of substance giving a formol titration. It is possible to fractionate commercial papain in this way so that about two-thirds of the material can be removed without appreciably impairing the activity of the remaining material.

Using the information obtained in the above experiments, about 50 gm. of papain were purified for use in the experiments given below.

150 gm. of commercial papain were rubbed with 4,500 cc. of water and allowed to stand over night. The next morning 4,000 cc. of clear solution were siphoned off and without filtering were poured into 8 liters of acetone. The precipitate was allowed to settle. After standing 4 hours the clear supernatant fluid was decanted off and the precipitate filtered and washed with acetone. The precipitate was finally drained on a large Buchner funnel and then rubbed up with 800 cc. of warm water, and the turbid brown solution allowed to stand 36 hours in a tall cylinder, a layer of toluene acting as a preservative. After standing, the clear supernatant liquid was siphoned off and poured into 4 liters of 95 per cent alcohol, and the precipitate filtered on a Buchner funnel. The filtration proceeded very slowly, taking 24 hours. The precipitate was rubbed with 95 per cent alcohol and then with ether, and dried after filtration in a current of air. The drying was rather unsatisfactory, the material becoming light brown in color. The activity of the material was, however, very high, so that it is certain that the ferment is fairly stable in aqueous solution and precipitated in the presence of acetone, alcohol, and ether. This observation is contradictory to some of the statements that appear in the literature regarding the deterioration of papain. Other experiments also confirmed the conclusion reached here. Papain allowed to stand over night at 37° seems to show little if any deterioration. Dialysis of the ferment in collodion bags results in a certain loss of activity, the bag contents becoming less active while the dialysate becomes slightly active, the sum of the two or the combined action of both being less than that of the untreated aqueous solution that stood under the same conditions. The deterioration is accelerated by dialyzing at 37°.

Optimal Hydrogen Ion Concentration for the Papain Action.

Inasmuch as most ferments seem to have a definite range of acidity or alkalinity in which they exhibit their maximal activity, it seemed strange that papain should, as stated in the literature, act equally well in acid or alkaline solution. To throw more light on this point a series of experiments was undertaken to determine at which hydrogen ion concentrations papain was most active proteolytically. The data recorded below are typical of the results obtained in different experiments so the conclusion seems justified that papain, in common with other ferments, has an optimal hydrogen ion concentration, in this case approximately 10^{-5} N. In all cases, the indicator method was used and the results are therefore not more accurate than a half a unit in the pH². In the presence of proteins the indicator results are not entirely to be relied upon except for comparative purposes. The absolute hydrogen ion concentrations of the various solutions used cannot be given with certainty.

A 2 per cent solution of gelatin in water was treated with HCl and NaOH so that the solutions when tested with suitable indicators showed that they were of the hydrogen ion concentration desired. A 0.5 per cent solution of purified papain was divided into three parts and adjusted to 10^{-3} , 10^{-6} , and 10^{-9} N. 25 cc. portions of the various gelatin solutions were measured out and treated with 5 cc. of the papain solution of the same range of acidity. Duplicate blanks were set up with the papain and the gelatin and triplicate mixtures were made containing the protein and ferment.

Proteolytic Action of Papain at Various Hydrogen Ion Concentrations.

pH.		Action.
Initial.	Final.	
2	2	0.50
3	3.5	1.45
4	4	4.95
5	5	5.35
6	6	3.35
7	6	2.55
8	6.5	1.50
9	7	1.25

² The symbol pH is used interchangeably with the term hydrogen ion concentration and denotes numerically the negative exponent of 10.

Of the latter, one was used to test the hydrogen ion concentration before and after the flasks were allowed to stand in the incubator. The period of incubation was 22 hours. The results given in the column under actions are the formol titrations in cc. of 0.1 N alkali after correcting for all blanks.

The above experiment shows fairly conclusively that papain exhibits its greatest activity at an acidity equal to the concentration of the hydrogen ion of 10^{-5} N; *i.e.*, slightly more acid than is necessary to cause methyl red to change from yellow to red. The method of experimentation used above gives results which are entirely in accord with those obtained when the rate of cleavage of gelatin and egg white is followed at different hydrogen ion concentrations. It is interesting to note the changes in hydrogen ion concentration that occur during the proteolysis. In those cases either side of the optimum acidity, the tendency is for the solution to become more acid or alkaline, apparently tending to bring the solution to the optimum acidity. This is rather peculiar in view of the fact that at all times the solutions contain a large quantity of material that might act as buffer. In fact as the digestion proceeds the buffer action should become more marked since a greater number of amino and carboxyl groups are present. The only explanation that is apparent at present must involve an assumption that postulates two different types of cleavage products, depending on the hydrogen ion concentration. In one case we must assume the liberation of a preponderance of basic amino-acids or peptides, in the other an excess of acid compounds.

Having found that there was a definite hydrogen ion concentration at which papain was most active proteolytically, the question to what extent the ferment was decomposed on standing with acid and alkali was raised. To throw light on this point, papain solutions were treated with different strengths of acid and alkali and then neutralized to methyl red (hydrogen ion concentration 10^{-5} N) and allowed to act on gelatin. The actions were compared with those of the untreated solution of papain at the same hydrogen ion concentrations. Suitable blanks for enzyme and substrate were run and the results corrected for them. Toluene was used as a preservative.

Influence of Acids and Alkalies on the Proteolytic Activity of Papain.

Concentration.	Time of standing.	Action.	Remarks.
	<i>hrs.</i>		
0.5 N acid.....	4	0.10	Incubation with gelatin 41 hrs.
0.1 " ".....	4	0.25	
Water.....	4	4.95	
0.1 N alkali.....	4	0.20	
0.5 " ".....	4	0.05	
0.05 N acid.....	1	2.30	Incubation with gelatin 18 hrs.
0.02 " ".....	1	2.90	
Water.....	1	4.10	
0.02 N alkali.....	1	3.65	
0.05 " ".....	1	2.85	

The above experiment shows that the ferment is sensitive to both acid and alkali, the latter being less destructive.

Quantitative Relationships between Papain and Its Substrate.

In studying the changes that occur when an enzyme and its substrate react it is evident that while the enzyme is affecting the substrate, the latter is modifying the activity of the enzyme. It has been stated in the literature of the subject that the proteolytic activity of enzymes follows the simple mass action law, this conclusion being deduced from the study of the kinetics of hydrolysis. There are several reasons why it is perhaps a fruitless task to try to formulate a statement of the kinetics of the reaction involving the enzymatic cleavage of a protein. First, the system involves two colloidal components and it is therefore unlikely that solution kinetics will apply, but instead adsorption phenomena may be the basic factors (Nelson and Vosburgh, 1917). Second, the cleavage of protein does not represent a reaction where one stage is completed before another begins but rather a complex of a number of simultaneous reactions.

In order to determine what rôle the relative quantities of enzyme and substrate play in the action of papain on protein the following experiment was carried out.

A series of solutions of gelatin of definite concentration were treated with acid until their hydrogen ion concentration as indicated colorimetric-

ally was 10^{-5} N. Similarly a series of papain solutions were prepared. Mixtures of these as indicated in the table were incubated at 37° for 24 hours and the extent of cleavage was determined by the formol titration. Toluene was added as a preservative. All the data recorded are corrected for suitable blanks on enzyme and substrate.

Proteolysis with Varying Concentrations of Enzyme and Substrate.

Papain.	Gelatin.			
	mg. 125	mg. 250	mg. 500	mg. 750
	Formol titration.			
mg.				
5	1.70	2.65	3.25	3.55
10	2.10	3.50	5.05	5.90
25	2.25	4.40	7.30	8.95
50	2.45	4.70	8.55	11.00

The results of the above experiment lend support to the view that in the cleavage of protein by papain there is a two stage reaction, the first involving a combination of enzyme and substrate, and the second the cleavage of this intermediate compound to give the enzyme and the split products of the protein. It will be seen from the curves that when the amount of the substrate present is relatively small, the proteolysis is not proportional to the enzyme concentration but tends to a definite point, the addition of further enzyme producing little additional cleavage. In the case where the ferment is kept constant, the proteolysis depends on the ratio of substrate to ferment. If the ferment concentration is large the "formol" titration after proteolysis is almost directly proportional to the quantity of substrate. With smaller substrate concentrations the action is dependent on the concentration up to a certain point, after which the addition of more substrate causes little more action. These findings would indicate that a given quantity of enzyme can handle a given quantity of substrate after which the addition of either component leads to no further action. This brings the action of papain into the same class as urease, invertase, and lipase. In considering the relations of enzyme and substrate, it appeared of interest to determine to what extent the addition of more enzyme

and substrate to a digestion mixture would affect the results. The results of the experiments are summarized in the following table. In the table indications are made of the amounts of ferment and substrate added on the 1st and 2nd day and the "formol" titrations of the resulting digestion mixture at the end of the 2nd day recorded under Action, the figure given being corrected for all blanks.

Influence of the Addition of Ferment and Substrate to a Digestion Mixture.

No.	1 per cent papain solution.		5 per cent gelatin solution.		Action.	Remarks.
	1st day.	2nd day.	1st day.	2nd day.		
1	cc. 5	cc. 0	cc. 5	cc. 0	2.40	
2	5	5	5	0	2.55	Slight increase due to additional quantity of ferment.
3	10	0	5	0	2.70	Twice the original quantity of ferment gives only slightly larger action than No. 2.
4	5	0	5	5	3.50	Addition of more substrate shows that active ferment remains but that action on 2nd day is smaller than on 1st.
5	5	0	10	0	5.30	Twice the original quantity of substrate gives much larger action than No. 4. Time factor may play a rôle.
6	5	5	5	5	4.35	Two individual additions of enzyme and substrate do not cause twice the action in No. 1, due possibly to a retarding effect of the products.

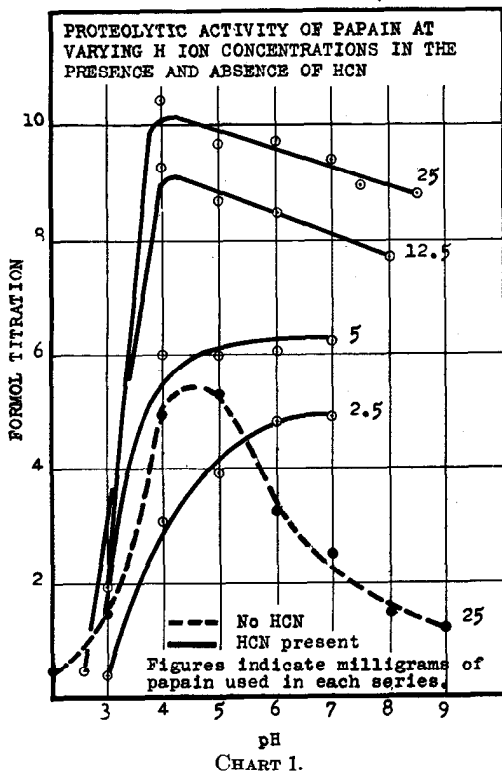
These experiments also support the view that there is a definite quantity of ferment required for a given amount of substrate and that an excess causes little more action.

The striking action of HCN on proteolytic activity of papain has been noted by Vines (1903) and Mendel and Blood (1910). The experiments of these authors were for the most part qualitative in character and served to show that HCN had a definite rôle as a specific activator of papain action, altering the character of the reaction to such an extent that more extensive cleav-

Proteolytic Activity of Papain-HCN at Varying Hydrogen Ion Concentrations with Varying Concentrations of Papain.

Experiment 1.			Experiment 2.			Experiment 3.			Experiment 4.		
Gelatin.....	500 mg.		Gelatin ..	500 mg.		Gelatin....	500 mg.		Gelatin.....	500 mg.	
HCN.....	53 "		HCN.....	53 "		HCN.....	67 "		HCN.....	53 "	
Papain.....	2.5 "		Papain.....	5 "		Papain....	12.5 "		Papain.....	25 "	
Volume.....	35 cc.		Volume....	35 cc.		Volume....	35 cc.		Volume.....	35 cc.	

pH.			pH.			pH.			pH.		
Initial.		Final.	Initial.		Final.	Initial.		Final.	Initial.		Final.
Action.			Action.			Action.			Action.		
						2.5	3.5	0.55	2	2	0.50
3	3.5	0.40	3	3.5	1.50				3	3.5	1.90
4	4.5	3.10	4	4.5	6.00	4	4	9.30	4	4	10.45
5	5	3.90	5	5	6.00	5	5	8.70	5	5	9.70
6	5.5	4.85	6	5.5	6.05	6	6	8.50	6	6	9.75
7	5.5	4.90	7	5.5	6.25				7	6	9.40
						8	7.5	7.70	7.5	6	8.95
									8.5	6.5	8.80



age of the protein resulted. In considering the nature of the active groupings in papain it seemed desirable to review some of the experiments of the earlier workers, using more modern methods and taking into consideration the factors of acidity and the quantitative relationships between the various components of the reacting system.

The optimal hydrogen ion concentration for papain activity in the presence of HCN was determined in the same way as in the case where no HCN was used, the same ferment solution being taken, under much the same conditions.

The data above are in marked contrast to those obtained where no HCN was present. Instead of finding a definite hydrogen ion optimum for papain-HCN proteolysis we find that the ferment is equally active, or nearly so, over a wide range of acidity. The results obtained with lower concentrations of ferment leave the whole matter unsettled. Further work on this point involving more careful measurements of the hydrogen ion concentration by means of the gas chain method is planned. The rôle of the HCN is not at all clear. From experiments presented below, it would appear that the HCN combines with the papain and the substrate to form an intermediate compound which then undergoes cleavage much as the intermediary compound of papain and protein does. Under such circumstances it may be that the ternary HCN-papain-protein compound has different stability.

To determine whether proteolysis in the system papain-protein-HCN follows the same general scheme as in the system papain-protein, the following set of experiments were carried out.

*Proteolysis with Varying Concentrations of Enzyme and Substrate.
HCN Constant.*

Papain.	Gelatin.			
	mg.	mg.	mg.	mg.
	125	250	500	750
	Formol titration.			
mg.				
5	2.55	4.50	7.50	9.60
10	2.75	4.95	8.80	12.10
25	3.10	5.75	10.45	14.45
50	3.35	6.25	11.45	16.25

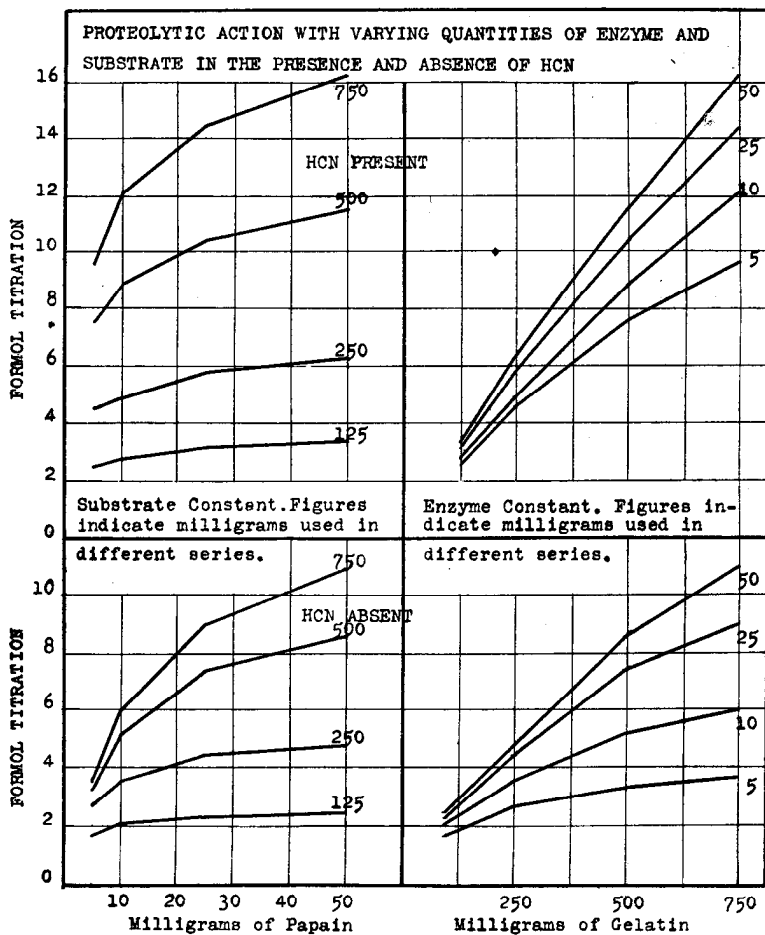


CHART 2.

The general plan was the same as before. The solutions of gelatin and papain were adjusted to pH 5 and the requisite amounts added to each experiment. The HCN solution used was prepared by dissolving 13 gm. of Kahlbaum's KCN in normal HCl, enough (200 cc.) of the latter being used to make the solution just neutral to methyl red. 2 cc. of this solution were used in each experiment. The volume of the digestion mixtures was 32 cc. Toluene was added as an additional preservative. The results given are corrected formol titrations.

To determine the relation between the extent of proteolysis and the amount of HCN used, the following experiment was

carried out. The same enzyme, substrate, and HCN solution were used as before, the quantities of the first two being kept constant while the third was varied. The results are given below.

*Proteolysis with Varying Quantities of HCN, Enzyme, and Substrate Constant.
Papain 10 Mg., Gelatin 750 Mg., Volume 30 Cc.*

HCN.	Formol titrations.		
	Titration.	Action due to HCN.	Action per unit HCN.
cc.			
0	5.30		
1	10.75	5.45	5.45
2	12.10	6.80	3.40
5	12.80	7.50	1.50
10	13.90	8.60	0.86

The data presented in the two tables above indicate that proteolysis in the system papain-HCN-protein follows the same general laws as were noted above for the papain-protein system. Fixing two components we find that increase of the third tends to increase the total cleavage but not in proportion to the amount added. In fact there seems to be a tendency towards a definite maximum. The only explanation of this phenomenon that is apparent is one which assumes the existence of an intermediary ternary compound in which all three components are present in definite ratio. Any excess of enzyme or HCN over that necessary to give the proper combination seems to remain in the system without taking part in the reaction. If an excess of substrate be present, it would seem as though some of the material were awaiting its turn to be used.

In the experiments of Mendel and Blood various attempts were made to explain the action of HCN in papain proteolysis. They found that among other things methyl cyanide did not have the same effect as HCN, indicating that it was not the nitrile group that was involved. They noted that KCN had less action than HCN but this is undoubtedly due to the fact that the alkalinity of the KCN gave rise to an unfavorable hydrogen ion concentration. The only other substance that was found to be effective in accelerating the action of papain on protein was hydrogen sulfide. This led them to suggest that possibly the reducing properties of the two substances were responsible for their action.

If the reducing properties of HCN were responsible for its activity in papain proteolysis it might be expected that some of the HCN would be destroyed in the course of the digestion. The following experiment was carried out to test this point.

5 gm. of gelatin were dissolved in 200 cc. of water containing 0.4 per cent of trichresol. The solution was divided into two equal parts and 10 cc. of 1 per cent HCN solution added to each. To one 50 mg. of papain were added, and the other was used as a control. Both flasks were incubated at 37° for 24 hours, and then acidified with 15 cc. of 15 per cent sulfuric acid and distilled in steam, the distillates being collected in alkaline solution. The distillates were titrated with silver nitrate according to Liebig's method for cyanide determination with the following results:

	AgNO ₃ cc.
10 cc. HCN solution.....	19.35
Control gelatin HCN.....	18.1
Papain HCN digestion.....	19.2

These results show clearly that HCN is not oxidized or converted into a compound that is not readily hydrolyzed by dilute acid. It is of course quite possible that the HCN enters into some combination that is not very stable in the presence of acid and can therefore be recovered completely with the method used. Further experiments on this point are in progress.

It has been claimed that in the course of papain proteolysis free amino-acids are liberated. Mendel and Blood obtained evidence of the formation of tryptophane in papain-HCN digestion. Abderhalden and Teruuchi (1906) claimed that the ferment could effect the cleavage of glycylytyrosine. In our experiments we have not found it possible to hydrolyze glycylyglycine, alanylglycine, glycylyalanine, alanyllalanine, or glycylytyrosine with papain either in the presence or absence of HCN. In the experiments on alanylglycine the hydrogen ion concentration was varied over a wide range with no change in the result. How far up in the scale it is necessary to go to effect cleavage with papain is as yet unknown. Some experiments undertaken from a different point of view may be of interest in this connection.

200 cc. portions of 1 per cent gelatin and dried egg albumin solutions were adjusted to pH 5 and treated with 75 mg. of papain. The solutions were incubated at 37° and 25 cc. portions were withdrawn at the intervals

noted and titrated by the formol method. The results given under Action are the formol titrations corrected for the titration of 25 cc. immediately on mixing the ferment with the substrate. When the solutions were nearly in equilibrium, several 25 cc. portions were withdrawn and treated with HCN. The formol titrations were then made after incubation for the stated period.

Gelatin.		Egg white.	
Time.	Action.	Time.	Action.
<i>hrs.</i>		<i>hrs.</i>	
1½	0.35	3	0.35
3	0.65	5	0.35
4½	0.80	22	0.75
23	2.00	46	1.15
69	2.80	70	1.30
HCN added.			
72	3.15	94	3.30
96	3.95		

These experiments indicate that HCN is effective in renewing the proteolytic activity of papain even after equilibrium is apparently reached. Whether this effect is due to a cleavage of compounds of lower molecular weight or of some unattacked protein is as yet unsettled. *A priori*, it would appear that the former view is correct. It is the plan of the author to investigate further this whole question of HCN action in certain fermentations since it offers a new point of attack in the study of the chemistry of these reactions.

SUMMARY

A method of purification of crude papain is presented.

The conditions of acidity for the optimum action of papain are found to be $\text{pH} = 10^{-5}$.

A consideration of the quantitative relations between papain and its substrate leads to the view that this ferment acts like urease, invertase, and lipase in forming an intermediary compound which is broken up into the cleavage products and liberates the enzyme.

Investigation of the action of HCN in papain hydrolysis leaves this question still unsettled. There seems to be some difficulty in defining a hydrogen ion optimum for papain-HCN proteolysis. The quantitative relations of the enzyme, HCN, and protein lend support to the view that there is a ternary intermediary compound formed by the components which then breaks down into cleavage products of the protein, enzyme, and HCN.

It has been shown that HCN may be recovered almost quantitatively from digestion mixtures, indicating that it is not utilized in the reaction of fermentation.

Papain, with or without HCN, seems to have no proteolytic effect on the dipeptides studied. HCN can renew proteolysis in papain digests that are almost in equilibrium.

BIBLIOGRAPHY.

- Abderhalden, E., and Teruuchi, Y., *Z. physiol. Chem.*, 1906, xlix, 21.
Mendel, L. B., and Blood, A. F., *J. Biol. Chem.*, 1910, viii, 177.
Vines, S. H., *Ann. Bot.*, 1903, xvii, 606.
Nelson, J. M., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1917, xxxix, 790.